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(54) Title: METHODS OF INDUCING A CYTOTOXIC IMMUNE RESPONSE AND RECOMBINANT SIMIAN ADENOVIRUS COMPOSITIONS USEFUL THEREIN

(57) Abstract: A method of inducing a CD8+ T response against a selected molecule by delivering the molecule via a recombinant simian adenovirus is provided. Also provided are methods of inducing interferon-α and interferon-β by delivering a recombinant simian adenovirus to a subject. The methods and compositions of the invention are particularly well suited for prophylaxis and treatment of infections with human immunodeficiency virus and human papilloma virus, among others, and cancer therapy.

METHODS OF INDUCING A CYTOTOXIC IMMUNE RESPONSE AND RECOMBINANT SIMIAN ADENOVIRUS COMPOSITIONS USEFUL THEREIN

BACKGROUND OF THE INVENTION

Adenoviral recombinants of the human serotype 5 have been tested as vaccine carriers for a variety of antigens derived from viruses, parasites or tumor cells. The results were encouraging as E1-deleted adenoviral recombinants elicited immune responses to the transgene product. Adenovirus of the human serotype 5 (Ad5) is a ubiquitous common-cold virus that infects most humans within their first year of life. The inventors have found that pre-existing immunity to common human serotypes reduce the efficacy of adenoviral recombinant vaccine based on the homologous serotype of virus. In some cases this reduction in efficacy can be overcome by delivery of higher doses of the human adenoviral recombinants. However, these higher doses may be associated with other undesirable side effects.

What are needed are compositions useful for inducing an immune response to a selected molecule, which avoid the problems associated with current delivery methods.

SUMMARY OF THE INVENTION

The present invention provides a method of preferentially inducing a cytotoxic immune response to a heterologous molecule by delivering the molecule to a host via a recombinant simian adenovirus. The inventors have unexpectedly found that recombinant simian adenoviruses, used according to the present invention, present an immunogen in a manner which induces a significantly more potent CD8+ T cell response than when the immunogen is delivered by a comparable human type 5 virus. In addition, the inventors have found that the recombinant chimpanzee adenoviruses

induce approximately five-fold higher levels of interferon-α and interferon-β than do the human adenoviruses.

Thus, in one aspect, the present invention provides a method of preferentially inducing a CD8+ T cell response to a heterologous molecule in a subject by delivering a recombinant simian adenovirus carrying the molecule to the subject. In one desirable embodiment, the recombinant simian adenovirus is a recombinant chimpanzee adenovirus strain.

In another aspect, the invention provides a method of inducing an interferon-α and/or interferon-β response in a subject by delivering a recombinant simian adenovirus to a subject.

In still another aspect, the invention provides an immunogenic composition useful for inducing a CD8+ T cell response against human immunodeficiency virus. The composition contains a recombinant simian adenovirus comprising an optimized nucleic acid sequence encoding a modified gag protein of human immunodeficiency virus-1 and a physiologically compatible carrier.

In yet another aspect, the invention provides a method for inducing a CD8+ T cell response against human immunodeficiency virus in mammals by administering to the mammal the immunogenic composition of the invention.

In a further aspect, the invention provides a method for inducing a CD8+ T cell response against human papilloma virus in mammals by administering to the mammal a recombinant simian adenovirus encoding an immunogenic protein derived from human papilloma virus.

Yet other advantages of the present invention will be readily apparent from the following detailed description of the invention.

Brief Description of the Drawings

Fig. 1 summarizes the genetic organization of the chimpanzee adenovirus C68 genome. In Fig. 1A the genome of the C68 chimpanzee adenovirus is schematically represented by the box at the top. The inverted terminal repeats are shaded black and the early regions are shaded gray. The arrowheads above the box indicate the

direction of expression of the early genes. The line below the box represents the division of the genome into 100 map units. The arrows below the line represent the five late gene regions and the proteins encoded in each region. The numbers below the box or arrows indicate the start (promoter or initiation codon) and end (canonical

5   PolyA signal) for each region. * represents the E2A late promoter. Fig. 1B illustrates the PstI clones; Fig. 1C illustrates the BamHI clones. Fig. 1D illustrates the HindIII clones. For parts 1B-1D, the unshaded regions indicate that a fragment was cloned into a plasmid vector, as listed in Table 1, while the shaded regions indicate that the restriction fragment was not cloned. For each section the fragment name, alphabetical

10   with A being the largest fragment, and the fragment size are listed above the box and the fragment end points are listed below the box.

Fig. 2 provides a multiple sequence alignment of hexon proteins. The deduced amino acid sequences of highly similar human adenovirus hexons were compared with the chimpanzee adenovirus using CLUSTAL X. Serotypes and subgroups are

15   indicated on the left margin, followed by the residue number. The numbering refers to the amino acid position with respect to the start of translation. Amino acids are shaded with respect to C68 to highlight sequence similarities (gray) and identities (black). The seven hypervariable regions within loop domains DE1 and FG1 are labeled along the bottom and correspond to the following Ad2 sequences in the

20   alignment: HVR1, 137-188; HVR2, 194-204; HVR3, 222-229; HVR4, 258-271; HVR5, 278-294; HVR6, 316-327; and HVR7, 433-465. The GenBank accession numbers for the sequences shown are as follow: AAD03657 (Ad4), S37216 (Ad16), S39298 (Ad3), AAD03663 (Ad7), and NP040525 (Ad2).

DETAILED DESCRIPTION OF THE INVENTION

25   In a comparison of the immunogenicity of the adenoviral recombinants of the human strain 5 to that of the chimpanzee adenovirus strain 68, both expressing a truncated sequence of gag, the chimp adenovirus was shown to be more potent. Similar results have been observed with recombinant chimpanzee adenoviruses expressing the green fluorescent protein and a rabies virus glycoprotein. This higher

potency of the recombinant chimpanzee adenovirus is most likely not linked to higher transgene expression as the studies were performed with both Ad and chimp recombinants carrying similar expression cassettes in which the transgene is controlled by the early cytomegalovirus promoter. The data presented herein also

5   indicates that it is unlikely to reflect differences in tropism, as both the chimp and Ad5 viruses utilize the same cellular receptor. Rather, these results demonstrate that the recombinant chimpanzee adenoviruses, used according to the present invention, unexpectedly have an adjuvanticity which differs from the human adenovirus. This better adjuvancy has a profound effect on the magnitude and kinetics of the transgene-

10   specific immune response induced by the chimp adenovirus.

Advantageously, this higher potency permits the use of lower doses of chimpanzee adenoviruses than would be required for a human adenoviral delivery system. In addition, the inventors have found that the recombinant chimpanzee adenoviruses induce approximately five-fold higher levels of interferon-α and

15   interferon-β than do the human adenoviruses.

Further, the recombinant chimpanzee adenoviruses have been found to have approximately the same ability upon dendritic cells as the human adenovirus type 5 viruses. This ability, coupled with the unexpected potency of the simian adenoviruses provides significant advantages in induction of a cytotoxic immune

20   response to a selected antigen and in the treatment of conditions for which enhanced induction of interferon-α and/or interferon-β are desirable.

I.   RECOMBINANT SIMIAN ADENOVIRUS
        A.   SOURCES

25   A variety of sources of chimpanzee adenovirus sequences are available from the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, and other sources. Desirable chimpanzee strains Pan 5 [ATCC VR-591], Pan 6 [ATCC VR-592], and Pan 7 [ATCC VR-593]. Particularly desirable chimpanzee adenovirus strains, are chimpanzee adenovirus strain Bertha or C1

30   [ATCC Accession No. VR-20] and chimpanzee adenovirus, strain Pan 9 or CV68

[ATCC VR-594]. For convenience, the virus CV68 is referred to throughout this specification as "C68". The viruses were originally isolated from feces [C1, Rowe et al, *Proc. Soc. Exp. Med.*, 91:260 (1956)] or mesenteric lymph node [C68, Basnight et al, *Am. J. Epidemiol.*, 94:166 (1971)] of infected chimpanzees. The sequences of these strains, and the location of the adenovirus genes E1a, E1b, E2a, E2b, E3, E4, L1, L2, L3, L4 and L5 are provided in US Patent 6,083,716, which is incorporated by reference herein. Optionally, non-chimpanzee simian adenoviral sequences may be used in preparing the recombinant vectors of the invention. Such non-chimpanzee adenovirus include those obtained from baboon adenovirus strains [e.g., ATCC VR-275], adenovirus strains isolated from rhesus monkeys [e.g., ATCC VR-209, ATCC VR-275, ATCC VR-353, ATCC VR-355], and adenovirus strains isolated from African green monkeys [e.g., ATCC VR-541; ATCC VR-941; ATCC VR-942; ATCC VR-943].

The recombinant chimpanzee (or other simian) adenoviruses described herein may contain adenoviral sequences derived from one, more than one simian adenoviral strain. These sequences may be obtained from natural sources, produced recombinantly, synthetically, or by other genetic engineering or chemical methods.

B.   RECOMBINANT SIMIAN ADENOVIRUSES

The recombinant simian adenoviruses useful in this invention are viral particles which are composed of recombinant simian adenoviruses sequences carrying a heterologous molecule and/or simian adenovirus capsid proteins. These simian adenoviruses, and particularly the chimpanzee C68 and C1 sequences are also useful in forming hybrid vectors with other simian and non-simian adenoviruses, and in forming pseudotyped recombinant viruses, i.e., recombinant viruses with an adenoviral vector carrying a heterologous molecule which is packaged in a heterologous capsid protein of simian origin.

1.   Recombinant Simian Adenovirus

At a minimum, a recombinant simian adenovirus useful in the invention contains the simian adenovirus cis-elements necessary for replication and virion encapsidation, which cis-elements flank the heterologous gene. That is, the

vector contains the cis-acting 5' inverted terminal repeat (ITR) sequences of the adenoviruses which function as origins of replication), the native 5' packaging/enhancer domains (that contain sequences necessary for packaging linear Ad genomes and enhancer elements for the E1 promoter), the heterologous molecule,

5   and the 5' ITR sequences. See, for example, the techniques described for preparation of a "minimal" human Ad vector in US Patent 6,203,975, which is incorporated by reference, can be readily adapted for the recombinant simian adenovirus.

        Optionally, the recombinant simian adenoviruses useful in this invention contain more than the minimal simian adenovirus sequences defined above.

10   These other Ad vectors can be characterized as having modifications which destroy the ability of the adenovirus to express one or more selected gene products. The phrase "functional deletion" is used herein to describe these modifications. Such "functional deletions" typically take the form of a deletion of all or a portion of a gene of the virus. However, such functional deletions may also take the form of a frame

15   shift mutation. Still other suitable manipulations which achieve functional deletion will be readily apparent to those of skill in the art.

        In a particularly desired embodiment, the simian adenoviruses are replication defective due to the absence of the ability to express adenoviral E1a and E1b, i.e., are functionally deleted in E1a and E1b. These recombinant simian

20   adenoviruses may also bear functional deletions in other genes.

        For example, the adenovirus delayed early gene E3 may be eliminated from the simian adenovirus sequence which forms a part of the recombinant virus. The function of E3 is not necessary to the production of the recombinant adenovirus particle. Thus, it is unnecessary to replace the function of

25   this gene product in order to package a recombinant simian adenovirus useful in the invention.

        Recombinant simian adenoviruses may also be constructed having a functional deletion of the E4 gene, although it may be desirable to retain the E4 ORF6 function. Still another vector of this invention contains a deletion in the

30   delayed early gene E2a. Deletions may also be made in any of the late genes L1

through L5 of the simian adenovirus genome. Similarly, deletions in the intermediate genes IX and IVa$_2$ may be useful for some purposes. Other deletions may be made in the other structural or non-structural adenovirus genes. The above discussed deletions may be used individually, i.e., an adenovirus sequence for use in the present invention

5   may contain deletions of E1 only. Alternatively, deletions of entire genes or portions thereof effective to destroy their biological activity may be used in any combination. For example, in one exemplary vector, the adenovirus sequence may have deletions of the E1 genes and the E4 gene, or of the E1, E2a and E3 genes, or of the E1 and E3 genes, or of E1, E2a and E4 genes, with or without deletion of E3, and so on. Such

10   deletions may be used in combination with other mutations, such as temperature-sensitive mutations, to achieve a desired result.

The transgene may be inserted into any deleted region of the simian adenovirus. Alternatively, the transgene may be inserted into an existing gene region to disrupt the function of that region, if desired.

15   Regardless of whether the recombinant simian adenovirus contains only the minimal Ad sequences, or the entire Ad genome with only functional deletions in the E1 and/or E3 regions, the recombinant virus contains a simian adenovirus capsid. Alternatively, in other embodiments, recombinant pseudotyped adenoviruses may be used in the methods of the invention. Such

20   pseudotyped adenoviruses utilize simian adenovirus capsid proteins in which a nucleic acid molecule carrying heterologous simian adenovirus sequences, or non-simian adenovirus sequences have been packaged. These recombinant simian adenoviruses of the invention may be produced using methods which are known to those of skill in the art.

25   C.   Production of the Recombinant Viral Particle

Methods of producing suitable recombinant simian adenoviruses utilize techniques which are well known to those of skill in the art, e.g., such as are described in US Patent 6,083,716. In the construction of recombinant simian adenoviruses for delivery of a heterologous molecule to a subject (e.g., a

human, canine, feline, or other mammalian), the adenovirus nucleic acid sequences employed in the vectors can be derived from a variety of simian sources.

A vector comprising simian (e.g., chimpanzee) adenovirus sequences which lacks simian adenovirus sequences necessary for production of an
5   infectious recombinant virus particle may be used in conjunction with a helper virus or vector. The helper virus provides essential gene products required for viral infectivity and propagation of the simian adenovirus. When only one or more selected deletions of simian adenovirus genes are made in an otherwise functional viral vector, the deleted gene products can be supplied in the viral vector production process by
10   propagating the virus in a selected packaging cell.

Thus, these functions may be provided in a permanently transformed cell line which provides some or all of the adenoviral functions which are required for packaging, e.g., any of the E1a, E1b, E2a, E4 ORF6, VA RNAs, which are lacking in the vector. If necessary or alternatively, any additional adenoviral
15   functions required may provided to the packaging cell by transfection or infection of a construct containing these functions. Optionally, the adenoviral functions may be selected to permit packaging of a viral vector carrying the minigene into a heterologous simian adenoviral capsid protein. Suitable methods of "pseudotyping" utilizing the simian (e.g., C68) capsid proteins will be readily apparent based upon
20   that which is known in the art regarding pseudotyping of human adenovirus. See, e.g., US Patent 6,203,975.

Assembly of the selected DNA sequences of the adenovirus, and the transgene and other vector elements into various intermediate plasmids and shuttle vectors, and the use of the plasmids and vectors to produce a recombinant viral
25   particle are all achieved using conventional techniques. Such techniques include conventional cloning techniques of cDNA such as those described in texts [Sambrook et al, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (1989)], use of overlapping oligonucleotide sequences of the adenovirus genomes, polymerase chain reaction, and any suitable method which
30   provides the desired nucleotide sequence. Standard transfection and co-transfection

techniques are employed, e.g., CaPO$_4$ precipitation techniques. Other conventional methods employed include homologous recombination of the viral genomes, plaquing of viruses in agar overlay, methods of measuring signal generation, and the like.

For example, following the construction and assembly of the

5   desired transgene-containing shuttle vector, the shuttle vector is transfected *in vitro* into the host cell for packaging. The host cell has, or is provided with, any missing adenoviral functions. Homologous recombination occurs between the helper and the vector sequences, which permits the adenovirus-transgene sequences in the vector to be replicated and packaged into virion capsids, resulting in the recombinant

10   adenoviral particles.

Advantageously, the inventors have found that the human adenovirus E1 proteins transcomplement the E1-defective simian adenovirus to permit its packaging into simian adenoviral particles. However, because of the low degree of   . homology between the human Ad E1 and the sequences flanking the deleted simian

15   Ad E1 sequences, there is minimal risk that the simian Ad E1 will homologously recombine to produce replication-competent simian adenovirus.

The recombinant simian adenoviral particles, so produced, may be isolated and purified by any of a variety of methods known to those of skill in the art for use in the method of the invention.

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II.   Heterologous Molecules for Delivery to a Host

A.   Immunogens

The heterologous molecule carried on the simian adenovirus for delivery to a host cell may be any desired substance including, without limitation, a

25   polypeptide, protein, enzyme, carbohydrate, chemical moiety, or nucleic acid molecule which may include oligonucleotides, RNA, DNA, and/or RNA/DNA hybrids. In one desirable embodiment, the molecule carried by the simian adenovirus is a transgene. The transgene a nucleic acid molecule comprising a nucleic acid sequence, heterologous to the adenovirus sequences, which encodes a protein, peptide,

30   polypeptide, enzyme, or another product of interest and regulatory sequences directing

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transcription and/or translation of the encoded product in a host cell, and which enable expression of the encoded product in the host cell or the subject. The composition of the transgene depends upon the intended use for the simian adenovirus.

For example, one type of transgene comprises a reporter or marker
5   sequence which, upon expression, produces a detectable signal. However, particularly desirable are gene products and other molecules which to which an antibody and, most desirably, cell-mediated immune response are induced.

These immunogenic gene products and molecules may be from a wide variety of pathogenic microorganisms, including but not limited to those from viruses,
10   bacteria, fungi or parasitic microorganisms which infect humans and non-human vertebrates, or from a cancer cell or tumor cell. The immunogen may comprise peptides or polypeptides derived from proteins. In some instances, more than one immunogen is included in the composition.

Desirable immunogenic compositions containing these gene products
15   and other molecules include those directed to the prevention and/or treatment of disease caused by, without limitation, viruses such as Human immunodeficiency virus, Simian immunodeficiency virus, Respiratory syncytial virus, Parainfluenza virus types 1-3, Influenza virus (e.g., influenza A and B viruses), Herpes simplex virus, Human cytomegalovirus, hepatitis viruses (including Hepatitis A, Hepatitis B,
20   and Hepatitis C viruses), Human papillomavirus, poliovirus, rotavirus, caliciviruses, Measles virus, Mumps virus, Rubella virus, adenovirus, rabies virus, canine distemper virus, rinderpest virus, coronavirus, parvovirus, infectious rhinotracheitis viruses, feline leukemia virus, feline infectious peritonitis virus, avian infectious bursal disease virus, Newcastle disease virus, Marek's disease virus, porcine respiratory and
25   reproductive syndrome virus, equine arteritis virus and various Encephalitis viruses.

Still other immunogens are directed to the prevention and/or treatment of disease caused by, without limitation, bacteria such as *Haemophilus influenzae* (both typable and nontypable), *Haemophilus somnus*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*,
30   *Streptococcus faecalis*, *Helicobacter pylori*, *Neisseria meningitidis*, *Neisseria*

gonorrhoeae, Chlamydia trachomatis, Chlamydia pneumoniae, Chlamydia psittaci,
*Bordetella pertussis, Salmonella typhi, Salmonella typhimurium, Salmonella
choleraesuis, Escherichia coli, Shigella, Vibrio cholerae, Corynebacterium
diphtheriae, Mycobacterium tuberculosis, Mycobacterium avium- Mycobacterium*

5   *intracellulare* complex, *Proteus mirabilis, Proteus vulgaris, Staphylococcus aureus,
Clostridium tetani, Leptospira interrogans, Borrelia burgdorferi, Pasteurella
haemolytica, Pasteurella multocida, Actinobacillus pleuropneumoniae* and
Mycoplasma gallisepticum.

Still other desirable immunogens are those directed to the prevention

10   and/or treatment of disease caused by, without limitation, fungal pathogens such as
Aspergillis, Blastomyces, Candida, Coccidiodes, Cryptococcus and *Histoplasma.*

In addition, other desirable immunogens are those directed to the
prevention and/or treatment of disease caused by, without limitation, parasites such as
Leishmania major, Ascaris, Trichuris, Giardia, Schistosoma, Cryptosporidium,

15   *Trichomonas, Toxoplasma gondii* and *Pneumocystis carinii.*

Further, desirable immunogens include those directed to eliciting a
therapeutic or prophylactic anti-cancer effect in a vertebrate host, such as, without
limitation, those utilizing a cancer antigen or tumor-associated antigen including,
without limitation, prostate specific antigen, carcino-embryonic antigen, MUC-1,

20   Her2, CA-125 and MAGE-3.

The examples provided below specifically illustrate the advantages of
the methods and compositions of the invention utilizing a recombinant simian
adenoviral vector from which an immunogenic peptide of rabies (glycoprotein G) or
human immunodeficiency virus-1 (a modified gag protein) is expressed. Another

25   desirable embodiment utilizes a simian adenovirus carrying an immunogenic peptide
from human papilloma virus. However, the invention is not limited to these sources
of immunogens.

B.   Regulatory Elements

Design of a transgene or another nucleic acid sequence that requires

30   transcription, translation and/or expression to obtain the desired gene product in cells

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and hosts may include appropriate sequences that are operably linked to the coding

sequences of interest to promote expression of the encoded product. "Operably

linked" sequences include both expression control sequences that are contiguous with

the nucleic acid sequences of interest and expression control sequences that act *in*

5   *trans* or at a distance to control the nucleic acid sequences of interest.

Expression control sequences include appropriate transcription

initiation, termination, promoter and enhancer sequences; efficient RNA processing

signals such as splicing and polyadenylation signals; sequences that stabilize

cytoplasmic mRNA; sequences that enhance translation efficiency (i.e., Kozak

10   consensus sequence); sequences that enhance protein stability; and when desired,

sequences that enhance protein secretion. A great number of expression control

sequences -- native, constitutive, inducible and/or tissue-specific -- are known in the

art and may be utilized to drive expression of the gene, depending upon the type of

expression desired. For eukaryotic cells, expression control sequences typically

15   include a promoter, an enhancer, such as one derived from an immunoglobulin gene,

SV40, cytomegalovirus, etc., and a polyadenylation sequence which may include

splice donor and acceptor sites. The polyadenylation (polyA) sequence generally is

inserted following the transgene sequences and before the 3' adenovirus ITR sequence.

In one embodiment, the bovine growth hormone polyA is selected. A simian

20   adenovirus of the present invention may also contain an intron, desirably located

between the promoter/enhancer sequence and the transgene. One possible intron

sequence is also derived from SV-40, and is referred to as the SV-40 T intron

sequence. Another element that may be used in the vector is an internal ribosome

entry site (IRES). An IRES sequence is used to produce more than one polypeptide

25   from a single gene transcript. An IRES sequence would be used to produce a protein

that contains more than one polypeptide chain. Selection of these and other common

vector elements are conventional and many such sequences are available (see, e.g.,

Sambrook et al, and references cited therein at, for example, pages 3.18-3.26 and

16.17-16.27 and Ausubel et al., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John

30   Wiley & Sons, New York, 1989).

In one embodiment, high-level constitutive expression will be desired. Examples of useful constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) (see, e.g.,

5   Boshart et al, *Cell*, **41**:521-530 (1985)), the SV40 promoter, the dihydrofolate reductase promoter, the β-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1α promoter (Invitrogen). Inducible promoters, regulated by exogenously supplied compounds, are also useful and include, the zinc-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse

10   mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No et al, *Proc. Natl. Acad. Sci. USA*, **93**:3346-3351 (1996)), the tetracycline-repressible system (Gossen et al, *Proc. Natl. Acad. Sci. USA*, **89**:5547-5551 (1992)), the tetracycline-inducible system (Gossen et al, *Science*, **268**:1766-1769 (1995), see also Harvey et al, *Curr. Opin. Chem. Biol.*,

15   **2**:512-518 (1998)), the RU486-inducible system (Wang et al, *Nat. Biotech.*, **15**:239-243 (1997) and Wang et al, *Gene Ther.*, **4**:432-441 (1997)) and the rapamycin-inducible system (Magari et al, *J. Clin. Invest.*, **100**:2865-2872 (1997)). Other types of inducible promoters which may be useful in this context are those which are regulated by a specific physiological state, e.g., temperature, acute phase, a particular

20   differentiation state of the cell, or in replicating cells only.

In another embodiment, the native promoter for the transgene will be used. The native promoter may be preferred when it is desired that expression of the transgene should mimic the native expression. The native promoter may be used when expression of the transgene must be regulated temporally or developmentally, or

25   in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further embodiment, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression.

Another embodiment of the transgene includes a transgene operably

30   linked to a tissue-specific promoter. For instance, if expression in skeletal muscle is

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desired, a promoter active in muscle should be used. These include the promoters
from genes encoding skeletal α-actin, myosin light chain 2A, dystrophin, muscle
creatine kinase, as well as synthetic muscle promoters with activities higher than
naturally-occurring promoters (see Li et al., *Nat. Biotech.*, 17:241-245 (1999)).

5   Examples of promoters that are tissue-specific are known for liver (albumin, Miyatake
et al. *J. Virol.*, 71:5124-32 (1997); hepatitis B virus core promoter, Sandig et al., *Gene
Ther.*, 3:1002-9 (1996); alpha-fetoprotein (AFP), Arbuthnot et al., *Hum. Gene Ther.*,
7:1503-14 (1996)), bone osteocalcin (Stein et al., *Mol. Biol. Rep.*, 24:185-96 (1997));
bone sialoprotein (Chen et al., *J. Bone Miner. Res.*, 11:654-64 (1996)), lymphocytes

10   (CD2, Hansal et al., *J. Immunol.*, 161:1063-8 (1998); immunoglobulin heavy chain; T
cell receptor α chain), neuronal such as neuron-specific enolase (NSE) promoter
(Andersen et al. *Cell. Mol. Neurobiol.*, 13:503-15 (1993)), neurofilament light-chain
gene (Piccioli et al., *Proc. Natl. Acad. Sci. USA*, 88:5611-5 (1991)), and the neuron-
specific vgf gene (Piccioli et al., *Neuron*, 15:373-84 (1995)), among others.

15   Of course, not all expression control sequences will function equally
well to express all of the transgenes of this invention. However, one of skill in the art
may make a selection among these expression control sequences without departing
from the scope of this invention. Suitable promoter/enhancer sequences may be
selected by one of skill in the art using the guidance provided by this application.

20   Such selection is a routine matter and is not a limitation of the molecule or construct.
For instance, one may select one or more expression control sequences may be
operably linked to the coding sequence of interest, and inserted into the transgene, the
minigene, and the transfer virus of the invention. After following one of the methods
for packaging the simian adenovirus taught in this specification, or as taught in the art,

25   one may infect suitable cells *in vitro* or *in vivo*. The number of copies of the minigene
in the cell may be monitored by Southern blotting or quantitative PCR. The level of
RNA expression may be monitored by Northern blotting or quantitative RT-PCR.
The level of expression may be monitored by Western blotting,
immunohistochemistry, ELISA, RIA, or tests of the gene product's biological activity.

30   Thus, one may easily assay whether a particular expression control sequence is

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suitable for a specific produced encoded by the transgene, and choose the expression
control sequence most appropriate. Alternatively, where the molecule for delivery
does not require expression, e.g., a carbohydrate, polypeptide, peptide, etc., the
expression control sequences need not form part of the recombinant simian

5   adenovirus or other molecule.

III.   FORMULATION OF VIRUS FOR DELIVERY
        The recombinant simian adenoviruses, preferably suspended in a
physiologically compatible carrier, may be administered to a human or non-human
mammalian patient. Suitable carriers may be readily selected by one of skill in the art

10   in view of the indication for which the transfer virus is directed. For example, one
suitable carrier includes saline, which may be formulated with a variety of buffering
solutions (e.g., phosphate buffered saline). Other exemplary carriers include sterile
saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil,
sesame oil, and water. The selection of the carrier is not a limitation of the present

15   invention.
        Optionally, the compositions of the invention may contain, in addition to the
recombinant simian adenovirus and carrier(s), other conventional pharmaceutical
ingredients, such as preservatives, chemical stabilizers, or for vaccine use, adjuvants.
Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic

20   acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and
parachlorophenol. Suitable chemical stabilizers include gelatin and albumin. Suitable
exemplary adjuvants include, among others, immune-stimulating complexes
(ISCOMS), LPS analogs including 3-O-deacylated monophosphoryl lipid A (Ribi
Immunochem Research, Inc.; Hamilton, MT), mineral oil and water, aluminum

25   hydroxide, Amphigen, Avirdine, L121/squalene, muramyl peptides, and saponins,
such as Quil A.

IV.   DELIVERY OF RECOMBINANT VIRUS FOR TREATMENT AND/OR
PROPHYLAXIS

The recombinant, replication defective adenoviruses are administered in a
"pharmaceutically effective amount", that is, an amount of recombinant adenovirus

5   that is effective in a route of administration to transfect the desired cells and provide
sufficient levels of expression of the selected gene to provide a therapeutic or vaccinal
immune response, e.g., some measurable level of protective immunity.

Conventional and pharmaceutically acceptable routes of administration
include, but are not limited to, intranasal, intramuscular, intratracheal, subcutaneous,

10   intradermal, rectal, oral and other mucosal and parental routes of administration. As
used herein, mucosal routes of administration include those which deliver to mucosal
tissues, including, without limitation, inhalation, oral, intranasal, vaginal, and rectal
routes. Routes of administration may be combined, if desired, or adjusted depending
upon the immunogen or the disease. For example, in prophylaxis of rabies, the

15   subcutaneous, intratracheal, intranasal and oral routes are preferred. The route of
administration primarily will depend on the nature of the disease being treated.

Doses or effective amounts of the recombinant replication-defective Ad virus
will depend primarily on factors such as the condition, the selected gene, the age,
weight and health of the animal, and may thus vary among animals.

20   Dosages of the viral vector will depend primarily on factors such as the
condition being treated, the age, weight and health of the patient, and may thus vary
among mammalian (including human) patients. Advantageously, the unexpected
potency of the recombinant simian (e.g., chimpanzee) adenoviruses of the invention
permits the use significantly lower amount of the recombinant chimpanzee adenovirus

25   to provide an effective amount to induce the desired immunogenic effect (e.g.,
induction of a predetermined level of CD8+ T cells). For example, an effective dose
of the recombinant simian adenovirus may be provided by 10^4 pfu and 10^6 pfu of the
chimpanzee adenovirus. However, higher doses may be readily selected, e.g.,
depending upon the selected route of delivery. For example, the viral vector may be

30   delivered in an amount which ranges from about 100 μL to about 100 ml, and more

preferably, about 1 mL to about 10 mL, of carrier solution containing concentrations of ranging from about 1×10^4 plaque forming units (pfu) to about 1×10^{13} pfu virus/ml, and about 1×10^9 to about 1×10^{11} pfu/ml virus, based upon an 80 kg adult weight. A preferred dosage is estimated to be about 50 ml saline solution at 2×10^{10}

5   pfu/ml. A preferred dose is from about 1 to about 10 ml carrier (e.g., saline solution) at the above concentrations. The therapeutic levels, or levels of immunity, of the selected gene can be monitored to determine the need, if any, for boosters. Following an assessment of CD8+ T cell response, or optionally, antibody titers, in the serum, optional booster immunizations may be desired. Optionally, the recombinant simian

10   adenoviruses may be delivered using a prime-boost regimen. A variety of such regimens have been described in the art and may be readily selected. One particularly desirable method is described in WO 00/11140, published March 2, 2000, incorporated by reference.

In one desirable embodiment, the invention provides a method of

15   preferentially inducing a CD8+ T cell response to a human immunodeficiency virus in a subject by delivering a recombinant simian adenovirus comprising a modified gag protein. The modified gag protein illustrated in the examples below has been optimized, e.g., as described in US Patent 5,972,596. The coding and protein sequences are reproduced herein in SEQ ID NO:6 and SEQ ID NO:7. See, also, G.

20   Meyers et al., Eds. *Human retroviruses and AIDS. A compilation and analysis of nucleic acid and amino acid sequences* (Los Alamos National Laboratory, Los Alamos, NM 1991). However, any of a variety of methods to improve expression of the gag protein, or any other selected immunogen or antigen as described herein, are known to those of skill in the art and may be utilized, e.g., humanization of the HIV-1

25   gag codon sequences, removal of the HIV-1 gag splice site, insertion of additional leader sequences upstream of the HIV-1 gag codon sequences, insertion of a Kozak sequence upstream of the HIV-1 gag codon sequences. The selection of the optimization method is not a limitation of the present invention. Alternatively, the method of the invention may be used to deliver a recombinant simian adenovirus

30   carrying an HIV envelope protein, or an HIV pol, to the subject. One desirable HIV

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envelope protein is HIV glycoprotein 120 for which sequences are available from GenBank. However, other suitable viral envelope proteins may be utilized. The sequence for HIV-1 pol is known, as are a variety of modified pol sequences. See, e.g., US Patent 5,972,596 and R. Scheider et al, *J. Virol*, 71(7):4892-4903 (July 1997).

5   In another desirable embodiment, the invention provides a method of preferentially inducing a CD8+ T cell response to a tumor-associated protein specific for a selected malignancy by delivering a recombinant simian adenovirus comprising a tumor-associated protein to the subject.. Such a protein includes cellular oncogenes such as mutated ras or p53.

10   In another embodiment, the invention provides a method of preferentially inducing a CD8+ T cell response to a tumor-associated protein specific for a selected malignancy by delivering a recombinant simian adenovirus comprising a tumor-associated protein to the subject

Still another desirable embodiment involves delivering a recombinant simian
15   adenovirus comprising a protein derived from human papilloma virus for prevention of infection therewith and for treatment and prophylaxis of associated conditions. For example, the protein may be selected from the group consisting of E6, E7 and/or L1 (Seedorf, K. *et al*, *Virol.*, **145**:181-185 (1985)). Where the condition is respiratory syncytial virus infection, the protein is selected from the group consisting of the
20   glyco- (G) protein and the fusion (F) protein, for which sequences are available from GenBank.

The following examples are provided to illustrate the invention and do not limit the scope thereof. One skilled in the art will appreciate that although specific reagents and conditions are outlined in the following examples, modifications
25   can be made which are meant to be encompassed by the spirit and scope of the invention.

Example 1 - Creation of an E1 deleted vector based on Chimpanzee
Adenovirus C68

A replication defective version of C68 was isolated for use in gene transfer.
The classic strategy of creating a recombinant with E1 deleted, by homologous

5   recombination in an E1 expressing cell line was pursued. The first step was creation
of a plasmid containing m.u. 0 through 1.3 followed by addition of a minigene
expressing enhanced green fluorescent protein (GFP) from a CMV promoter and C68
sequence spanning 9-16.7 m.u. This linearized plasmid was cotransfected into an E1
expressing cell line with Ssp I-digested C68 plasmid (SspI cuts at 3.6 m.u. leaving

10   4644 bp for homologous recombination). Experiments were initially conducted with
293 cells which harbor E1 from human Ad5 with the hope that this would suffice for
transcomplementation. Indeed, plaques formed which represented the desired
recombinant. The resulting vector was called C68-CMV-GFP.

The strategy for generating recombinants was modified to enable efficient and

15   rapid isolation of recombinants. First, the alkaline phosphatase DNA in the initial
shuttle vector was replaced with a prokaryotic GFP gene driven by the prokaryotic
promoter from lacZ. This allowed efficient screening of bacterial transformations
when attempting to incorporate a desired eukaryotic RNA pol II transcriptional unit
into the shuttle vector. The resulting transformation can be screened for expression of

20   GFP; white colonies are recombinants while green colonies are residual parental
plasmid.

A green-white selection has been used to screen the products of cotransfection
for the isolation of human Ad5 recombinants (A.R. Davis et al, Gene Thera., 5:1148-
1152 (1998)); this was adapted to the C68 system. The initial shuttle vector was

25   revised to include extended 3' sequences from 9 to 26 MU. This vector was
cotransfected with viral DNA from the original C68-CMV-GFP isolate that had been
restricted with Xba I, which cuts at MU 16.5 allowing for 9.5 Kb of overlap for
homologous recombination. The resulting plaques were screened under a phase
contrast fluorescent microscope for non-fluorescing isolates that represent the desired

recombinants. This greatly simplified screening in comparison to the standard methods based on structure or transgene expression.

A.   Shuttle Plasmid

To construct a plasmid shuttle vector for creation of recombinant C68
5   virus, the plasmid pSP72 (Promega, Madison, WI) was modified by digestion with Bgl II followed by filling-in of the ends with Klenow enzyme (Boehringer Mannheim, Indianapolis, IN) and ligation with a synthetic 12 bp Pac I linker (New England Biolabs, Beverly, MA) to yield pSP72-Pac. A 456 bp Pac I/SnaB I fragment spanning map unit (m.u. or MU) 0-1.3 of the C68 genome was isolated from the pNEB-BamE
10   plasmid containing BamHI E fragment of the C68 genome and cloned into Pac I and EcoR V treated pSP72-Pac to yield pSP-C68-MU 0-1.3. A minigene cassette consisting of the cytomegalovirus early promoter driving lacZ with a SV40 poly A signal was separated from pCMVβ (Clontech, Palo Alto, CA) as a 4.5 kb EcoRI/SalI fragment and ligated to pSP-C68-MU 0-1.3 restricted with the same set of enzymes,
15   resulting in pSP-C68-MU 0-1.3-CMVLacZ.

For the initial step in the isolation of the 9-16.7 MU region of C68, both pGEM-3Z (Promega, Madison, MI) and pBS-C68-BamF were double-digested with BamHI and Sph I enzymes. Then the 293 bp fragment from pBS-C68-BamF was ligated with pGEM-3Z backbone to form pGEM-C68-MU 9-9.8. A 2.4 kb fragment
20   including the C68 MU 9.8-16.7 was obtained from the pBS-C68 BamHB clone after XbaI digestion, filling in reaction and subsequent BamHI treatment and cloned into BamHI/SmaI double digested pGEM-C68-MU 9-9.8 to generate pGEM-C68-MU 9-16.7. The C68 9-16.7 m.u. region was isolated from pGEM-C68-MU 9-16.7 by digestion with EcoRI, filling in of the ends with Klenow enzyme (Boehringer
25   Mannheim, Indianapolis, IN), ligation of a synthetic 12 bp HindIII linker (NEB) and then digestion with HindIII. This 2.7 kb fragment spanning the C68 MU 9-16.7 was cloned into the HindIII site of pSP-C68-MU 0-1.3-CMVlacZ to form the final shuttle plasmid pC68-CMV-LacZ. In addition, an 820 bp alkaline phosphatase (AP) cDNA fragment was isolated from pAdCMVALP (K. J. Fisher, et al., *J. Virol.*, 70:520-532

(1996)) and exchanged for lacZ at Not I sites of pC68-CMV-lacZ, resulting in pC68-CMV-AP.

B.   Construction of Recombinant Virus

To create the E1-deleted recombinant C68-CMVEGFP vector, a pC68-

5   CMV-EGFP shuttle plasmid was first constructed by replacing the lacZ transgene in pC68-CMV-lacZ with the enhanced green fluorescent protein (EGFP) gene. The replacement cloning process was carried out as the follows. An additional NotI restriction site was introduced into the 5' end of the EGFP coding sequence in the pEGFP-1 (Clontech, Palo Alto, CA) by BamHI digestion, filling in reaction and

10   ligation of a 8 bp synthetic NotI linker (NEB). After NotI restriction of both constructs, the EGFP sequence was isolated from the modified pEGFP-1 and used to replace the lacZ gene in the pC68-CMV-lacZ. The pC68-CMVEGFP construct (3 µg) was co-transfected with Ssp I-digested C68 genomic DNA (1 µg) into 293 cells for homologous recombination as previously described (G. Gao, et al, *J. Virol*, 70:8934-

15   8943 (1996)). Green plaques visualized by fluorescent microscopy were isolated for 2 rounds of plaque purification, expansion and purification by CsCl gradient sedimentation (G. Gao, et al, cited above).

In an attempt to apply the convenient green/white selection process (A. R. Davis, et al., *Gene Thera.*, 5:1148-1152 (1998)) to construction of recombinant

20   C68 vectors, a 7.2 kb fragment spanning 9 to 36 MU was isolated from the pBSC68-BamB plasmid by treatment with AgeI and BsiwI restriction endonucleases and cloned into Asp718 and AgeI sites of pC68-CMV-AP shuttle plasmid, resulting in a new plasmid called pC68CMV-AP-MU36. A further modification was made to remove 26 to 36 m.u. from pC68CMV-AP-MU36 by Eco47III and NruI digestions.

25   The new shuttle plasmid called pC68CMV-AP-MU26 has a shorter region for homologous recombination (i.e., 16.7-26 MU) 3' to the minigene. To make a recombinant C68 vector, alkaline phosphatase (AP) is replaced with the gene of interest. The resulting pC68CMV-Nugene-MU26 construct is co-transfected with Xba I (16.5 MU) restricted C68-CMVGFP viral DNA into 293 cells, followed by top

30   agar overlay. The recombinant virus plaques (white) are generated through the

homologous recombination in the region of 16.7-26 MU which is shared between pC68CMV-Nugene construct and C68 viral backbone; the recombinants which form white plaques are selected from green plaques of uncut C68-CMVGFP virus.

5   The green/white selection mechanism was also introduced to the process of cloning of the gene of interest into the pC68 shuttle plasmid. The AP gene in both pC68CMV-AP-MU36 and pC68CMV-AP-MU26 was replaced with a cassette of prokaryotic GFP gene driven by the lacZ promoter isolated from pGFPMU31 (Clontech, Palo Alto, CA). Thus, white colonies of bacterial transformants will contain the recombinant plasmid. This green/white selection process for bacterial

10   colonies circumvented the need for making and characterizing large numbers of minipreped DNAs and so further enhanced the efficiency in creating recombinant C68 vectors.

Example 2 - Expression of Antigen (Gag secretion) in TK⁻ Cells Infected with Simian Adenovirus Vaccine Constructs

15   Adenoviral recombinants of the chimpanzee strain 68 (Adchimp68) and the human strain 5 (Adhu5) carrying a nucleotide sequence modified version of a truncated form of the gag gene of HIV-1 clade B were constructed as described (in Example 1 and Z.Q. Xiang, et al, *Virol.* **219**, 200 (1996)). Transcripts of structural proteins of HIV-1, including gag, contain genetic instability elements, which require

20   the presence of rev protein for nuclear export and efficient expression in the cytoplasm (S. Schwartz *et al., J Virol* **66,** 7176 (1992); S.Schwartz, et al, *J Virol 66,* 150-159 (1992); G. Nasioulas *et al., J Virol* **68**, 2986 (1994)). Adenoviruses rely on nuclear transcription and thus require rev for expression of HIV-1 proteins. To circumvent Rev dependency, a codon-modified sequence of gag from which genetic

25   instability elements had been removed by site directed mutagenesis (R. Schneider *et al., J Virol* **71**, 4892 (1997); S. Schwartz *et al., J Virol* **66**, 7176 (1992); S. Schwartz, et al., J Virol **66,** 150 (1992)) was inserted into the adenoviral vector. The introduced gene encodes the truncated p37gag protein (p17 and p24 regions). The truncated gag protein does not form viral particles and is partially secreted into the supernatant of

transfected human cells (R. Schneider *et al., J Virol* **71**, 4892 (1997)). The mutated gag constructs have been used in vaccination experiments and result in the generation of cellular and humoral immune responses in mice and primates (J.T. Qiu *et al. J Virol* **73**, 9145. (1999)).

5   The Adchimp68 and the Adhu5 recombinants were both generated and propagated on 293 cells transfected with the E1 of adenovirus of the human strain 5. The inventors have found that this heterologous E1 is suitable for complementing the E1-deleted Adchimp68 virus recombinants thus reducing the risk of recombination and reversion to replication-competent wild-type virus.

10   The presence of gag protein in the TK⁻ culture supernatants was analyzed by Western blotting using mouse monoclonal antibodies to gag. TK⁻ cells (1 x 10^6) were infected for 48 hrs with Adhu5gag37 or Adchimp68gag37 virus (10 pfu per cell). Additional TK⁻ cells were infected with an Adhu5 or an Adchimp68 construct expressing the glycoprotein of rabies virus. Proteins in the culture supernatant were

15   separated on a 12% denaturing polyacrylamide gel and transferred by electroblotting to a PVDF membrane. The blot was stained with the monoclonal antibody 183-H12-5C to HIV-1 p24 (B. Chelsebro, *et al.* J. Virol. **66**: 6547 (1992).

           The two adenoviral recombinant clones (Adhu5gag37, Adchimp68gag37) carrying this modified sequence of gag expressed the transgene product at comparable

20   levels as shown by Western Blot analysis. A protein of the expected size (37 kDa) that bound to a monoclonal antibody to gag of HIV-1 was detected in the supernatants of TK⁻ cells infected with 10 plaque forming units (pfu) of either adenovirus gag recombinant. Control cells infected with Adhu5 or Adchimp68 recombinant expressing glycoprotein of rabies virus (Adhu5rab.gp and Adchimp68.gp) failed to

25   produce this protein.

Example 3 - Induction of CD8$^+$ T cell responses to gag in mammals by Simian Adenovirus

           The following experiment demonstrates that the splenocytes of mice injected intramuscularly (i.m.) with either the Adhu5gag37 or the Adchimp68gag37

recombinant responded to an immunodominant epitope (B. Doe and C.M. Walker, *AIDS* **10**, 793 (1996)) of the gag protein by cytokine, i.e., interferon (IFN)-γ, release, as well as by target cell lysis.

A.   Cytokine Release Assay

5   Groups of 3 Balb/c mice were immunized i.m. with 2×10^5, 2×10^6 or 2×10^7 pfu of Adchimp68gag37 virus, 2×10^6 pfu of Adhu5L1 virus (H.C.J. Ertl, *et al., J. Virol*, 63:2885 (1989)), 2×10^6 pfu of Adhu5 gag37 virus or 2×10^7 pfu of VVgag virus. Splenocytes were tested for CD8$^+$ T cell response to gag 10 days later. To assay cytokine (IFN-γ) production, splenocytes (1×10^6/sample) were cultured for

10   5 hrs at 37°C with 3 μg/ml of the AMQMLKETI peptide (SEQ ID NO:1) which carries the immunodominant CD8$^+$ T cell epitope for the H-2^d haplotype and 1μg/ml Brefeldin A (GolgiPlug, PharMingen, San Diego, CA) in 96 well round-bottom microtiter plate wells in Dulbeccos modified Eagles medium (DMEM) supplemented with 2% fetal bovine serum (FBS) and 10^{-6}M 2-mercaptoethanol. Cells were washed

15   with PBS and incubated for 30 min at 4°C with a FITC labeled antibody to murine CD8. Cells were washed and permeabilized in 1X Cytofix/Cytoperm (PharMingen) for 20 min at 4°C. Cells were washed 3 times with Perm/Wash (PharMingen) and incubated in the same buffer for 30 min at 4°C with a PE labeled antibody to murine IFN-γ. After washing, cells were examined by two-color flow cytometry and data

20   were analyzed by WinmDi software. The number in the right hand corner shows the percent of CD8$^+$ cells over all CD8$^+$ T cells that stained positive for INF-γ.

         Seven to ten days after a single immunization, a sizable fraction of the entire splenic CD8$^+$ T cell population produced IFN-γ in response to the gag peptide. Primary splenocytes assayed without further in vitro expansion lysed H-2 compatible

25   target cells pre-treated with the gag peptide. Gag-specific CD8$^+$ T cell activity was superior upon immunization with the Adchimp68 construct, which achieved CD8$^+$ T cell frequencies to gag of ~ 16-19% of the entire splenic CD8$^+$ cell population. The response was dose-dependent as shown for the Adchimp68gag37 virus where a low dose of 2×10^5 pfu of virus still elicited frequencies of nearly 10%. The Adhu5gag37

30   recombinant induced optimal frequencies of ~9% at 2×10^6 pfu. These frequencies

were not significantly enhanced upon increasing the dose of this vaccine (data not shown). A vaccinia virus recombinant expressing full-length gag (VVgag, designated vDK1 in S. Chacarabarti et al. *Mol. Cell. Biol.* 5, 3403 (1985)) stimulated far lower frequencies of CD8$^+$ T cells by intracellular cytokine staining.

5   B.   Lysis of target cells.

Splenocytes from mice immunized 10 days previously with a single dose of the adenoviral recombinants as described in A or two doses of the VVgag recombinant the first given i.m. followed 2 weeks later by an intraperitoneal injection were tested in a 5 hr ^{51}Cr-release assay at varied effector to target cell ratios on 1 x 10^4

10   P815 cells that had been treated for 16-24 hrs at room temperature with either the peptide to gag (filled squares) or the control peptide 31D (X) delineated from the sequence of the rabies virus nucleoprotein (H.C.J. Ertl *et al.*, *J. Virol.* 63 : 2885 (1989)). Two immunizations with the VVgag vaccine were required to induce detectable T cell-mediated gag-specific primary cytolysis.

15   C.   Kinetics of the CD8$^+$ T cell response to gag

Groups of 4 Balb/c mice were immunized with 5 x 10^6 pfu of Adhu5gag37 or Adchimp68gag37 virus. Splenocytes were harvested 6-12 days later and tested for IFN-γ production and target cell lysis as described above. The kinetics of the CD8$^+$ T cell response to gag elicited by the two adenovirus recombinants

20   differed. The response to gag presented by the Adhu5gag37 virus peaked 2 - 4 days earlier than the CD8$^+$ T cell response to the Adchimp68gag37 recombinant.

Example 4 - Effect of Prior Exposure to Human Adenovirus on Simian Adenovirus Vaccine

To study the impact of previous exposure to the common human strain 5 of

25   adenovirus, mice were immunized with a single dose of an Adhu5 recombinant expressing an irrelevant antigen (human papilloma virus L1). Two weeks later mice were vaccinated either with the Adhu5gag37 or the Adchimp68gag37 vaccine.

More particularly, mice were immunized i.m. with 10^8 pfu of the Adhu5L1 vaccine. Two weeks later Adhu5-immune as well as naïve mice were injected with 2 x

10^6 or 2 x 10^7 pfu of Adhu5gag37 or Adchimp68gag37 recombinants (4-5 mice per group). Additional groups of Adhu5L1 immune or naïve mice were immunized with 2 x 10^6 pfu of the Adhu5gag37 or the Adchimp68gag37 virus. Nine days later mice were injected intraperitoneally with 10^6 pfu of a vaccinia virus recombinant

5   expressing full-length gag. Mice were sacrificed five days after the vaccinia virus injection.

Mice pre-immune to Adhu5 virus failed to respond to gag after vaccination with the Adhu5gag37 vaccine. They showed frequencies of CD8^{+} gag-specific T cells similar to those seen in control mice and correspondingly, their splenocytes failed to

10   lyse gag expressing target cells. In contrast, the CD8^{+} T cell response to gag was only slightly decreased in Adhu5-immune mice vaccinated with the Adchimp68gag37 construct. Frequencies of CD8^{+} T cells to gag were reduced by only ~ 30% and the cytolytic activity of splenocytes was lowered by ~ 50% comparing different effector to target cell ratios.

15   Thus, both adenoviral recombinants induce frequencies of CD8^{+} T cells to gag, surpassing those elicited by previously described vaccines such as naked DNA or poxvirus recombinants (S.Schwartz, et al, *J Virol 66*, 150-159 (1992)). Frequencies were also higher than those generally seen in chronically infected individuals (D.H. Barouch *et al. Proc. Natl. Acad. Sci USA.* **97**, 4192 (2000); T.U. Vogel *et al. J.*

20   *Immunol.* **164**, 4968 (2000); P.A. Goepfert *et al. J. Virol.* **74**, 10249 (2000); C.R. Rinaldo Jr. *et al.* AIDS Res. & Hum. Retr.. 14:1423 (1998)). These results emphasize the potency of adenoviral recombinant vaccines.

Example 5 - Effect of Priming and Boosting of CD8^{+} T cells to Antigen

Primary splenocyctes from the cells of naïve or Adhu5-immune mice

25   immunized with 2 x 10^7 pfu of Adhu5gag37 or Adchimp68gag37 virus were compared with splenocytes from naïve or Adhu5 immune mice vaccinated with 2 x 10^6 pfu of Adhu5gag37 or Adchimp68gag37 virus and then boosted with 10^6 pfu of VVgag virus. Splenocytes were analyzed 5 days later for CD8 and intracellular IFN-γ. These assays were performed essentially as described above, with the exception that

there was no further *in vitro* culture for lysis of P815 cells treated with the gag peptide or the control peptide 31D in a 5 hr ^{51}Cr-release assay.

Priming or booster immunization with a heterologous vaccine construct, the VVgag recombinant, failed to restore the CD8 T cell response to gag presented by the Adhu5 recombinant vaccine. Although Adhu5 vaccinated animals boosted with Adhu5gagp37 and VVgag showed as much as 7.1 % of splenic CD8$^+$ T cells to produce IFN-γ-in response to the gag these CD8$^+$ T cells totally lacked cytolytic activity against gag-presenting target cells. These results indicate that pre-exposure to the antigens of the vaccine carrier had not only a quantitative but also a qualitative influence on the CD8$^+$ T cell response to the transgene product of the adenoviral recombinant. The CD8$^+$ T cell response to gag in Adhu5 immune mice vaccinated with Adchimp68gag37 showed a booster effect upon VVgag immunization similar to that seen in naïve mice.

Frequencies of CD8$^+$ T cells to gag as well as primary target cell lysis could be augmented further by priming (not shown) or boosting with a heterologous vaccine carrier, such as the VVgag recombinant. After i.m. priming with the adenoviral recombinants followed 9 days later by an i.p. booster immunization with the VVgag, CD8$^+$ gag-specific T cells analyzed 5 days post-prime comprised ∼ 40% of the entire splenic CD8$^+$ cell-population.

Pre-existing immunity to Adhu5 severely reduced the efficacy of the Adhu5gag37 vaccine but only slightly impaired the CD8$^+$ T cell response to the Adchimp68gag37 virus. It was previously reported that mice immunized to Adhu5 virus developed a reduced B cell response to vaccination with an Adhu5 vaccine to rabies virus. Increasing the dose of the vaccine or using a DNA vaccine expressing the same antigen of rabies virus could readily circumvent the dampening effect of the pre-exposure to Adhu5 virus (Z.Q. Xiang, et al., *J. Immunol.* **162**, 6716 (1999)).

In contrast, the CD8$^+$ T cell response to gag presented by the Adhu5 recombinant vaccine was abolished in Adhu5 immune mice and could only partially be restored by additional immunizations with a heterologous vaccine to gag. This may

indicate induction of CD8$^+$ T cells to be more susceptible to interference by circulating virus neutralizing antibodies as compared to stimulation of B cells.

Example 6 - Production of Recombinant Adenoviruses Containing Rabies Glycoprotein

5   Adenoviruses of the human serotypes 2,4, 5, 7, 12 and the chimpanzee serotype 68 were propagated and titrated on human 293 cells. The recombinant adenoviruses based on the human serotype 5 expressing the glycoprotein of the ERA serotype of rabies virus or the L1 protein of the human papilloma virus (HPV)-16 have been described previously (Z. Q. Xiang, *et al*, *Virology* **219**: 220-227 (1996);

10   D. W. Kowalcyk, *et al*, (2001) Vaccine regimen for prevention of sexually transmitted infections with human papillomavirus type 16. *Vaccine*). An expression system based on adenoviruses of the chimpanzee serotype 68 was developed as described in Example 1.

       Adenoviruses were propagated on E1 (derived from the human serotype 5)-

15   transfected 293 cells (F. L. Graham, *et. al., J. Gen. Virol.* **36**: 59-74 (1977)). Viruses were harvested by freeze thawing of the cells. For some experiments virus was purified by CsCl gradient purification. For other experiments, cleared supernatant of the infected cells necrotized through three rounds of freeze thawing was used. Viruses were titrated on 293 cells to determine plaque forming units (pfu).

20   The adenoviral recombinant of the chimpanzee 68 serotype expressing the rabies virus glycoprotein, termed Adchimp68rab.gp was generated in 293 cells transfected with E1 of adenovirus human serotype 5 as described in detail in this example. Viral clones were initially screened by indirect immunofluorescence with the monoclonal antibody 509-6 to a conformation-dependent epitope of the rabies

25   virus glycoprotein. Upon selection of a stable adenoviral subclone, expression of full-length rabies virus glycoprotein by the Ad.chimp68rab.gp virus in infected TK$^-$ cells was confirmed by immunoprecipitation, as described in the following example.

Example 7 - Expression of the transgene product by the adenoviral recombinants.

This example shows that the Adhu5 virus achieved markedly higher levels of rabies virus glycoprotein expression in TK⁻ cells as compared to the Adchimp68
5   construct. Transcript levels for this transgene paralleled protein expression indicating that the difference was unrelated to differences in post-translational modifications. TK⁻ cells are CAR positive and rates of transduction by the viral serotypes should thus be comparable.

For use in these experiments, mammalian cells, i.e., baby hamster kidney
10   (BHK)-21 cells, E1-transfected 293 cells and TK⁻ cells, were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine, Na-pyrovate, non-essential amino acids, HEPES buffer, antibiotic and 10% fetal bovine serum (FBS).

A.   Immunoprecipitation

15   TK⁻ cells (10^6 per sample) were infected with 5 pfu per cell of either the rabies virus glycoprotein expressing adenoviral recombinants or control constructs expressing an unrelated viral antigen. After 48 hrs cells were washed twice with sterile phosphate buffered saline (PBS) and then incubated for 90 min in serum-free medium prior to the addition of 20 μl of ^{35}S-labeled cystein and methionin (Promix,
20   NEN, Boston, MA). After 4 hrs incubation, cells were washed with PBS and then treated for 20 min with 1 ml of protease inhibitors containing RIPA buffer. Cells and cell debris were removed from the wells, vortexed briefly and centrifuged for 2 min at 12.000 rpm. The supernatant was incubated for 90 min at 4°C with 15 μl/ml of ascitic fluid containing the 509-6 monoclonal antibody to the rabies virus glycoprotein.
25   Protein Sepharose G was added at 75μl per sample and incubated at 4°C under mild agitation for 30 min. The samples were pelleted by centrifugation and washed 4 times with RIPA buffer. The pellets were resuspended in 80 μl of loading buffer, boiled for 4 min. Samples (20 μl) were then separated over a 12% SDS-polyacrylamide (PAGE) gel in comparison to a molecular weight standard. Gels were dried onto filter papers

which were exposed for 48 hrs to a Kodak Scientific Imaging Film (X-Omat Blue XB-1).

The Adchimp68rab.gp recombinant expressed a protein of the expected size that bound to the 509-6 antibody. The precipitate of TK⁻ cells infected with the Adhu5rab.gp virus showed a band of the identical size that was absent in lysates from cells infected with adenoviral recombinants expressing an unrelated transgene product. Expression of the rabies virus glycoprotein was more pronounced in cells infected with the Adhu5rab.gp construct. The difference in expression of the transgene product may reflect pre-translational events such as differences in viral uptake, rate of transcription or transcript stability. Alternatively, translational or post-translational differences such as distinct side chain modifications may result in quantitative differences in serologically detectable protein.

To further distinguish between these two possibilities, the total RNA was isolated from TK⁻ cells infected with either of the adenoviral recombinants. Reverse transcribed mRNA to the rabies virus glycoprotein and a housekeeping gene was amplified by real-time PCR performed as described in part B.

    B.   Real time reverse transcription polymerase chain reaction
          (PCR)

Confluent monolayers of TK⁻ cells were infected in duplicate samples with 10 pfu of either of the adenoviral recombinants. Cells were isolated 24 hrs later and RNA was extracted with the TRI reagent according to the manufacturer's instructions (Mol. Res. Center, Cincinnati, OH). The RNA was treated with RNAse-free DNAse, purified by phenol extraction and adjusted to 50 ng of RNA per sample. The RNA was reverse transcribed and amplified with the Light Cycler-RNA amplification kit SYBR green (Roche, Mannheim, Germany; Z. He, et al, *Virology* **270**: 146-1617 (2000)) using primers for the rabies virus glycoprotein (SEQ ID NO:2: 5' AA GCA TTT CCG CCC AAC AC; SEQ ID NO:3: 3' GGT TAG TGG AGC AGT AGG TAG A) and the housekeeping gene glutaraldehyde-3-phosphate dehydrogenase (GAPDH) (SEQ ID NO: 4: 5' GGT GAA GGT CGG TGT GAA CGG ATT T; SEQ ID NO:5: 3' AAT GCC AAA GTT GTC ATG GAT GAC C).

The data in Table 1 provides the results. The data show the mean values for duplicate measurements ± SD.

Table 1

Source of RNA	Relative Transcript Quantity		
	GAPDH	rab.gp	Ratio (GAPDH/rab.gp)
TK⁻, Adhu5rab.gp	3.2±.2	3494±18	1082
TK⁻, Adchimp68rab.gp	0.52±.01111	64± 6	64

As shown by this data, the transgene transcripts adjusted to those of the housekeeping gene showed a quantitative difference comparable to that of serologically detectable protein.

In data not provided in this example, two other Adchimp68 recombinants expressing the green fluorescent protein and a codon-modified truncated gag protein of the human immunodeficiency virus-1 were compared to the Adhu5 recombinants expressing the same transgene products showed equivalent protein expression levels in TK⁻ cells. From this it has been concluded that the reduced expression of the rabies virus glycoprotein by the Adchimp68 virus reflects a difference neither in viral uptake nor in rate of transcription, which in both constructs is regulated by the same control elements.

Example 8 - Immunization of mice using a rabies virus antigen.

The rabies virus-specific antibody response to the Ad.chimp68rab.gp virus was compared to that of the Adhu5rab.gp virus in inbred and outbred strains of mice. Mice were injected with serial dilutions of either of the recombinants given s.c. or i.n. Sera were harvested 14 days later and tested for antibodies to the rabies virus glycoprotein by an ELISA and a virus neutralization assay. Adenoviral recombinants expressing an unrelated transgene, i.e., the gag of HIV-1 (described in the Examples

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above) were used as controls. These recombinants failed to induce an antibody

response to rabies virus detectable by either assay. A more detailed discussion of this

study and the results follows.

Female 6-8 week old C3H/He and C57Bl/6 mice were purchased from

5   Jackson Laboratory, Bar Harbor Maine. Outbred ICR mice were purchased from

Charles River (Wilmington, MA).

Mice were injected with varied doses of the adenoviruses or the

adenoviral recombinants given in 100 μl of saline subcutaneously (s.c.) or in 50 μl

intranasally (i.n.). Mice were challenged with rabies virus of the CVS-11 strain given

10   at 10 mean lethal doses (LD$_{50}$) intracerebrally (i.c.). Rabies virus of the Evelyn

Rokitniki-Abelseth (ERA) and the Challenge Virus Standard (CVS)-11 strain were

propagated on BHK-21 cells. ERA virus was purified over a sucrose gradient,

inactivated by treatment with betapropionolactone and adjusted to a protein

concentration of 0.1 mg/ml. CVS-11 virus was titrated on BHK-21 cells and by

15   intracerebral injection of adult ICR mice (Z.Q. Xiang, Z.Q. & H.C. Ertl, *J. Virol.*

Meth. **47**: 103-16 (1994)). Upon challenge mice were checked every 24-48 hrs for at

least 21 days. They were euthanized once they developed complete hindleg paralysis,

which is indicative of terminal rabies virus encephalitis.

The serological assays included enzyme linked immunoadsorbant assay

20   (ELISA), isotype profile of antibodies, and virus neutralization assays.

A.   ELISA

Mice were bled a varied time intervals after immunization by

retro-orbital puncture. Sera were prepared and tested for antibodies to rabies virus on

plates coated with 0.1 μg/well of inactivated rabies virus. Sera were tested for

25   antibodies to adenovirus on plates coated with 0.1 μg/well of purified E1-deleted

adenovirus recombinants to GFP of the human serotype 5 or the chimpanzee serotype

68. ELISAs were performed basically as described before (Z. Q. Xiang, *et al*,

Virology **219**, 220-227 (1996)). Plates were coated over night. They were then

blocked for 24 hrs with PBS containing 3% of bovine serum albumin (BSA). After

30   washing, sera diluted in PBS-3% BSA were added for 60 min. After washing, a 1:100

dilution of alkaline phosphatase conjugated goat anti mouse Ig (Cappel) was added for 1 hr on ice. After washing, substrate was added for 20-30 min at room temperature. Optical density was read at 405 nm.

B.   Isotypes of antibodies

Isotypes of antibodies to rabies virus were determined by an ELISA on plates coated with inactivated ERA virus using the Calbiochem Hybridoma Subisotyping (LaJolla, CA) kit with some minor previously described modifications (Xiang, Virol, 1996, cited above).

The isotype profile of antibodies to also differed upon s.c. immunization but was comparable upon i.n. application of the two adenoviral vaccines. Both recombinants, upon delivery by either route of inoculation, elicited IgG2a antibodies to the antigen of rabies virus.

Both recombinants upon i.n. immunization and the Adhu5rab.gp vaccine upon s.c. administration induced a pronounced IgG1 response indicative of Th2 help, which was lacking in the response to the Ad.chimp68rab.gp construct given s.c.

C.   Neutralizing Antibodies

Sera were tested for neutralizing antibodies to rabies virus of the CVS-11 strain, which is antigenically closely related to the ERA strain (Z. Q. Xiang, et al, *Virology.* **214**: 398-404 (1996)). A WHO reference serum was used for comparison. Titers are expressed as International Units.

The Adchimp68rab.gp virus given s.c. induced a less potent B cell response to the transgene product as compared to the Adhu5rab.gp construct. The difference in magnitude of the antibody response, which was observed at all time points tested depended on the mouse strain and was less pronounced in outbred ICR than in inbred C3H/He mice. In contrast, upon i.n. immunization both vaccines induced comparable titers of antibodies as determined by ELISA and by virus neutralization assay.

The pronounced Th1 response to the Adchimp68rab.gp recombinant upon s.c. immunization contrasting with the more balanced Th1/Th2

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response upon injection of the Adhu5rab.gp argues for a difference in adjuvanticity. Upon application to the airways, the natural route of infection for Adhu5 virus and presumably for Adchimp68 viruses both recombinants induced antibody titers to the transgene product that were comparable in magnitude and in their isotype profile. This

5 suggests that postulated differences in tropism and/or adjuvanticity are tissue dependent, i.e., lacking or less pronounced in the airways as compared to the subcutaneum.

Example 9 - Preferential Induction of Cytotoxic T Cell Response with Recombinant Chimpanzee Adenovirus

10   Vaccine-induced protection to rabies virus correlates with virus-neutralizing antibodies (VNAs, F. L. Graham, et. al., J. Gen. Virol. **36**, 59-74 (1977)). The studies with the rabies protein thus focused on stimulation of this arm of the immune system. Throughout all of the experiments, mice were immunized with the Adchimp68rab.gp virus and, in parallel, with the previously described Adrab.gp virus

15 based on the human serotype 5. Within this application, this recombinant is referred to as Adhu5rab.gp virus.

          Both adenoviral recombinants induced protection to challenge with rabies virus. C3H/He mice immunized with 5×10^6 pfu of either of the adenoviral recombinants given s.c. remained disease-free when challenged 3 weeks later with 10

20 mean lethal doses (LD_{50}) of rabies virus of the CVS strain. This rabies virus strain is antigenically closely related to the ERA strain but is more virulent in rodents. At a lower vaccine dose of 5×10^5 pfu, the Adhu5rab.gp virus still provided complete protection while a small percentage of Adchimp68rab.gp-immunized mice succumbed to the infection. Further reduction of the vaccine dose resulted in loss of efficacy of

25 the Adchimp68rab.gp vaccine. Upon i.n. immunization, both vaccines provided complete protection if given at 5×10^5 pfu. At a lower dose of 5×10^4 pfu 50% of mice vaccinated with the Adhu5rab.gp vaccine developed progressive disease while those immunized with this dose of the Adchimp68rab.gp recombinant were protected. All of the mice immunized with adenoviral recombinants of either serotype

expressing an unrelated antigen or with 5 x 10³ pfu of either of the adenoviral
recombinants to the rabies virus glycoprotein developed a fatal rabies encephalitis.

Example 10 - The Effect of Pre-Existing Immunity to Different Serotypes of
Human Adenoviruses on the Antibody Response to Rabies Virus.

5   To test if pre-exposure to any of the common serotypes of human adenoviruses
(e.g., human serotype 2, 4, 5, 7 and 12) would inhibit the antibody response to the
Adchimp68rab.gp vaccine, groups of C3H/He mice were immunized with 4 x 10⁸ pfu
of replication-competent adenoviruses of the human serotypes 2, 4, 5, 7 or 12 or the
chimpanzee serotype 68 (the latter serotype was E1-deleted). Two weeks later, mice
10   were vaccinated s.c. with either Adhu5rab.gp or Adchimp68rab.gp virus. The
Adhu5rab.gp recombinant was used at a dose of 2 x 10⁵ pfu per mouse, the
Adchimp68rab.gp recombinant, which given s.c. only induces a marginal antibody
response in C3H/He mice at such a low dose was injected at 2 x 10⁷ pfu per mouse.
Sera were harvested 2 weeks later and tested for antibodies to the rabies virus
15   glycoprotein by an ELISA. The rabies virus-specific response to Adhu5rab.gp was
slightly superior in naïve mice to that elicited to the Adchimp68 virus. The response
to Adhu5rab.gp virus was completely inhibited in Adhu5 pre-immune mice. Some
reduction was also seen in mice pre-immune to adenovirus of the human serotypes 4,
2, 7 and 12. The response was not affected in mice that had been pre-exposed to the
20   Adchimp68 virus. The response to the Adchimp68rab.gp virus was strongly inhibited
in mice that were pre-immune to the homologous virus. Mice that had previously
encountered adenovirus of the human serotype 2 showed a slight reduction of the
antibody response to the rabies virus antigen presented by the Adchimp68 vaccine.
Mice inoculated with any of the other serotypes of human adenoviruses developed
25   antibody titers to rabies upon Adchimp68rab.gp virus that were either similar or
increased in magnitude compared to those in mice that were naïve prior to
vaccination. In particular, mice pre-immune to Adhu5 virus developed higher
antibody titers upon vaccination with the Adchimp68rab.gp construct which might

reflect the presence of cross-reactive T helper cells that promoted the B cell response to the transgene product.

To further determine if at equal vaccine doses the Adchimp68rab.gp vaccine induced superior antibody titers as compared to the Adhu5rab.gp virus in mice pre-immune to Adhu5 virus, a vaccine titration experiment was conducted. Groups of C3H/He mice were immunized s.c. with 4×10^8 pfu of an E1-deleted adenoviral recombinant to the L1 antigen of HPV-16. Mice were vaccinated 2 weeks later with either Adhu5rab.gp or Adchimp68rab.gp virus given s.c. at varied doses. Mice were bled 2 weeks later and serum antibody titers to rabies virus were determined by an ELISA (not shown) and a virus neutralization assay. Neither assay showed a significant reduction for the antibody response to the Adchimp68rab.gp construct in Adhu5-immune mice. The severity of the reduction of antibody titers to rabies virus presented by the Adhu5 construct in mice pre-immune to the homologous virus depended on the vaccine dose. The antibody response to lower doses of vaccine was more affected than the response to higher vaccine doses. VNA titers were substantially more reduced than the ELISA titers. Titers of VNAs to the highest vaccine dose were halved in mice pre-immune to Adhu5 virus while at the two lower vaccine doses titers were reduced by more than 20 fold. At any of the doses tested, the Adchimp68rab.gp recombinant induced higher VNA titers to rabies in Adhu5 pre-immune mice compared to those achieved by an equal dose of the Adhu5rab.gp vaccine. The detrimental effect of pre-existing immunity to Adhu5 on the efficacy of the Adhu5 vaccine was demonstrated further in a protection experiment. Naïve mice immunized with 2×10^5 pfu of Adhu5rab.gp or Adchimp68rab.gp virus were completely protected to challenge with CVS-11 virus. The majority (65%) of Adhu5 pre-immune mice immunized with this dose of the Adhu5rab.gp vaccine succumbed to a rabies virus infection while those vaccinated with the same dose of the Adchimp68rab.gp virus remained protected. Increasing the dose of the Adhu5rab.gp virus to 2×10^6 pfu per mouse restored the efficacy of the vaccine.

The antibody response to the transgene product expressed by the Adchimp68 recombinant was not affected by pre-existing immunity to common

human adenovirus serotypes, which inhibits the response to the corresponding
recombinant of the human serotype 5. Upon pre-immunization with replication-
competent viruses, the immune response to the Adhu5rab.gp vaccine was abolished in
Adhu5 pre-immune mice and reduced in mice pre-immune to other human serotypes

5   of adenovirus such as 2 and 4. The response to the Adchimp68 recombinant was as
expected inhibited in mice pre-immune to the homologous virus. This is not of
clinical concern as Adchimp68 virus does not circulate in the human population and
common human serotypes do not share neutralizing epitopes with Adchimp68 virus.

             Pre-exposure to replication-defective Adhu5 virus also reduced the

10   antibody response to the rabies virus glycoprotein presented by the Adhu5
recombinants although the impact was not as severe as in mice previously infected
with replication-competent virus. Sera from mice pre-immune to replication-defective
Adhu5 virus developed reduced but readily detectable antibodies to rabies virus upon
immunization with the Adhu5rab.gp vaccine. Increasing the dose of the Adhu5rab.gp

15   construct could in part circumvent the impact of pre-existing immunity. Vaccine-
induced protection against rabies virus requires VNAs, which were not induced as
efficiently in pre-immune mice by the Adhu5 vaccine especially when used at lower
doses. In Adhu5 pre-immune mice the VNA response to the Adchimp68rab.gp
construct was superior at all doses tested to that of the Adhu5 vaccine thus more than

20   compensating for the slightly lower potency of this vaccine upon s.c. immunization.

             Adchimp68 recombinants thus provide an attractive alternative as a
vaccine carrier for use in humans. As shown here they are efficacious even when
applied at low doses of 2×10^5 pfu through non invasive routes of administration
such as the upper airways. Mucosal immunization by i.n. application has the added

25   advantage of favoring induction of responses of the common mucosal immune
system, which is distinct from, albeit interconnected with the central immune system
targeted by injected vaccines.

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Example 11 - Chimpanzee C68 Virus Stock and Replication

Examples 11 through 15 which follow provide additional characterization of the chimpanzee C68. It will be appreciated by one of skill in the art that this information can be readily used in the construction of novel recombinant chimpanzee

5   adenoviral constructs.

The C68 virus stock was obtained from ATCC (Rockville, MD) and propagated in 293 cells (ATCC) cultured in DMEM (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS; Sigma or Hyclone, Logan, UT) and 1% Penicillin-Streptomycin (Sigma). Infection of 293 cells was carried out in DMEM

10   supplemented with 2% FCS for the first 24 hours, after which FCS was added to bring the final concentration to 10%. Infected cells were harvested when 100% of the cells exhibited virus-induced cytopathic effect (CPE), collected, and concentrated by centrifugation. Cell pellets were resuspended in 10mM Tris (pH8.0), and lysed by 3 cycles of freezing and thawing. Virus preparations were obtained following 2 ultra

15   centrifuge steps on cesium chloride density gradients and stocks of virus were diluted to 1 x 10^{12} particles/ml in 10mM Tris/100mM NaCl/50% glycerol and stored at -70°C.

Example 12 - Cloning and sequencing of viral genomic DNA

Genomic DNA was isolated from the purified virus preparation following

20   standard methods and digested with a panel of 16 restriction enzymes following the manufacturer=s recommendations. Except as noted, all restriction and modifying enzymes were obtained from Boehringer Mannheim, Indianapolis, IN. Genomic DNA was digested with BamHI, PstI, SalI, HindIII or XbaI and the fragments were subcloned into plasmids (K. L. Berkner and P.A. Sharp, *Nucl. Acids Res.*, 11:6003-20

25   (1983)). After deproteination, synthetic 10bp PacI linkers (New England Biolabs, Beverly, MA) were double digested with PacI and BamHI, or PstI.

The PstI, BamHI and HindIII clones generated from C68 are illustrated in Figure 1, parts C, D and E, respectively. The fragments indicated by the shaded boxes were not cloned, but the sequence of the entire genome has been determined through

sequencing overlapping clones and viral DNA directly (unshaded boxes). The cloned fragments are described in Table 2.

Table 2. C68 plasmid clones and insert sizes

Construct Name	Insert Size (base pairs)	Fragment 5' End	Fragment 3' End	5' End Map Unit	3' End Map Unit
Pst-I Fragments					
C68-Pst-A	6768	24784	31551	67.9%	86.4%
pBS:C68-Pst-B	6713	4838	11550	13.2%	31.6%
pBS:C68-Pst-C	5228	14811	20038	40.6%	54.9%
pBS:C68-Pst-D	2739	12072	14810	33.1%	40.6%
pBS:C68-Pst-E	2647	20039	22685	54.9%	32.1%
pBS:C68-Pst-F	1951	32046	33996	87.8%	93.1%
pNEB:C68-Pst-G	1874	1	1874	0.0%	5.1%
pBS:C68-Pst-H	1690	23094	24783	63.2%	67.9%
pBS:C68-Pst-I	1343	33997	35339	93.1%	96.8%
pNEB:C68-Pst-J	1180	35340	36519	96.8%	100.0%
pBS:C68-Pst-K	1111	2763	3873	7.6%	10.6%
pBS:C68-Pst-L	964	3874	4837	10.6%	13.2%
pBS:C68-Pst-M	888	1875	2762	5.1%	7.6%
pBS:C68-Pst-N	408	22686	23093	62.1%	63.2%
C68-Pst-O	380	31666	32045	86.7%	87.7%
pBS:C68-Pst-P	285	11551	11835	31.6%	32.4%
C68-Pst-Q	236	11836	12071	32.4%	33.1%
pBS:C68-Pst-R	114	31552	31665	86.4%	86.7%
BamHI Fragments					
C68-Bam-A	16684	19836	36519	54.3%	100.0%
pBS:C68-Bam-B	8858	3582	12439	9.8%	34.1%
pBS:C68-Bam-C	4410	12440	16849	34.1%	46.1%
pBS:C68-Bam-D	2986	16850	19835	46.1%	54.3%
pNEB:C68-Bam-E	2041	1	2041	0.0%	5.6%
pBS:C68-Bam-F	1540	2042	3581	5.6%	9.8%
HindIII Fragments					
pBR:C68-Hind-B	9150	23471	32620	64.3%	89.3%

pBS = pBluescript SK+ clone

pNEB = pNEB 193 clone

pBR = pBR322 clone

No prefix = fragment not cloned

Chimpanzee adenovirus, C68, was obtained from ATCC and propagated in human 293 cells. Viral genomic DNA was isolated from purified virions using established procedures (A. R. Davis, *et al.*, *Gene Thera.*, 5:1148-1152 (1998)) and digested with a panel of restriction enzymes; the data were consistent with previous

5   studies (data not shown) (G. R. Kitchingman, *Gene*, 20:205-210 (1982); Q. Li and G. Wadell, *Arch Virol.* 101:65-77 (1998); R. Wigand, *et al.*, *Intervirology.* 30:1-9 (1989)). Restriction fragments spanning the entire genome of C68 were subcloned into plasmids. A schematic drawing of the C68 genome is shown in Figure 1A, and the Pst-I, BamHI and HindIII fragments that were cloned into plasmid vectors are

10   indicated by the unshaded boxes, in Figs. 1B, 1C, and 1D, respectively. The cloned fragments, fragment sizes and genomic position are also listed in Table 2. Both plasmid clones and genomic DNA were used as templates for sequencing. The genome was sequenced by primer walking in both directions and each base was included in an average of approximately four reactions.

15   The C68 genome is 36521 bp in length [see, US Patent 6,083,716]. Preliminary comparison with GenBank sequences indicated varying degrees of similarity with other human and animal adenoviruses along the entire length of the viral genome. Regions with homology to all of the previously described adenoviral genetic units, early regions 1-4 and the major late genes, were found in the C68

20   genome (Fig. 1A). DNA homology between C68 and the human adenoviruses that have been completely sequenced, Ad2 (NC001405), Ad5 (NC001405), Ad12 (NC001460), Ad17 (NC002067) and Ad40 (NC01464), was used to order the clones. The open reading frames (ORF) were determined and the genes were identified based on homology to other human adenoviruses. All of the major adenoviral early and late

25   genes are present in C68. The inverted terminal repeats (ITR=s) are 130 bp in length.

Example 13 - Analysis of C68 sequence

    The complete nucleotide sequence of every member of the Mastadenovirus genus accessible from GenBank, including isolates from different species, were screened for identity to C68. The Ad4 minigenome was assembled from the

following GenBank sequences: Left-hand ITR (J01964); E1A region (M14918); DNA

pol and pTP (X74508, 74672); VA RNA-I, II (U10682); 52, 55K (U52535); pVII

(U70921); hexon (X84646); endoprotease (M16692); DNA-binding protein

(M12407); fiber (X76547); right-hand ITR (J01965). The Ad7 composite genome

5   was created from the following sequence data: Mu 3-21 (X03000); VA RNA-I, II,

pTP & 52, 55K (U52574); penton (AD001675); pVI, hexon and endoprotease

(AF065065); DNA-binding protein (K02530); E3 and fiber region (AF104384); right-

hand ITR (V00037).

The amino acid sequence alignment was generated with Clustal X, edited with

10   Jalview (http://www.ebi.ac.uk/~michele/jalview/), and analyzed with Boxshade

(http://www.ch.embnet.org/software/BOX_form.html). Publicly available hexon

protein sequences from all human adenovirus serotypes were initially aligned to

identify the set showing the highest homology to C68.

The nucleotide sequence and predicted amino acid sequences of all significant

15   open reading frames in the C68 genome were compared to known DNA and protein

sequences. The nucleotide sequence of C68 is compared to sequences of Ad 2, 4, 5,

7, 12, 17 and 40. In agreement with previous restriction analysis (Kitchingman, cited

above; Li and Wadell, cited above) C68 is most similar to human Ad4 (subgroup E).

The E1A region of C68 extends from the TATA box at nt 480 to the poly A

20   addition site at 1521. The consensus splice donor and acceptor sites are in the

analogous position of the human Ad counterparts, and the 28.2K and 24.8K proteins

are similar in size to the human Ad proteins. The ORF for the smallest E1A protein

of C68 is predicted to encode 101 residues as opposed to approximately 60 amino

acids for other adenoviruses. There is a TTA codon at residue 60 for C68 where other

25   adenoviruses often have a TGA stop codon. The first 60 residues of C68 E1A 100R

protein have 85% identity to the Ad4 homolog.

The C68 genome encodes genes for the four E1B proteins, 20.5K, 54.7K,

10.1K and 18.5K as well as pIX. All five C68 encoded proteins are similar in size to

that of other Ad E1B and pIX proteins. The Ad4 homolog of the E1B 21K protein has

30   only 142 amino acids, where C68 has 186 residues and other human adenoviruses

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have 163-178 residues. The C68 and Ad4 proteins share 95% identity over the first

134 aa, then the similarity ends and the Ad4 protein terminates at 142 amino acids.

The C68 genome encodes homologs of the E2A 55K DNA binding protein and

the Iva2 maturation protein, as well as the E2B terminal protein and the DNA

5   polymerase. All of the E2 region proteins are similar in size to their human Ad

counterparts, and the E2B proteins are particularly well conserved. The C68 E2B

123.6K DNA polymerase is predicted to be 1124 residues, while Ad4 is predicted to

have 1193 although the other human adenoviruses have smaller polymerases.

Residues 1-71 of the Ad4 polymerase have no similarity to any other Ad polymerase,

10   and it is possible that this protein actually initiates at an internal ATG codon. From

amino acids 72-1193, Ad4 and C68 polymerases have 96% amino acid identity.

The E3 regions of human adenoviruses sequenced so far exhibit considerable

sequence and coding capacity variability. Ad40 has five E3 region genes, Ad12 has

six, C68 and Ad5 have seven, Ad38 has eight and Ad3 as well as Ad7 (subgroup B

15   human adenoviruses) have nine putative E3 region genes. The Ad4 E3 region has not

yet been sequenced. In comparison with the E3 region of Ad35, all 7 E3 gene

homologs were identified in the C68 genome (C. F. Basler and M.S. Horwitz,

Virology, **215**: 165-177 (1996)).

The C68 E4 region has 6 ORFs and each is homologous to proteins in the

20   human Ad5, 12 and 40 E4 region. The E4 nomenclature is confusing because the

ORF2 homologs of C68, Ad12 and Ad40 are approximately 130 residues, while in

Ad5 there are two ORFs encoding proteins of 64 and 67 residues with homology,

respectively, to the amino and carboxy terminal ends of the larger ORF2 proteins.

ORF5 has been omitted in our nomenclature because the 5th ORF in the E4 region is

25   homologous to the widely studied ORF6 protein of human Ad5.

The major late promoter and the tri-partite leader sequences of the C68

genome were located. ORFs with the potential to encode the 15 major late proteins

were located. All of the C68 late proteins are similar in size to their human Ad

counterparts. The percent amino acid identity between chimpanzee and human Ad

30   late proteins varies considerably. The C68 fiber protein is predicted to have 90%

amino acid identity with the Ad4 protein, but much less similarity to the other human
Ad fiber proteins. The CAR binding site in the fiber knob is present in C68.

Example 14 - Virus neutralizing antibody assays

Several studies were performed to determine if there is cross-reactivity

5   between type specific antisera of C68 and human adenovirus. The neutralizing
activity of sera was tested as follows. Panels of sera from normal human subjects
(N=50), rhesus monkeys (N=52) and chimpanzees (N=20) were evaluated for
neutralizing antibodies against Ad5 and C68 based vectors using 293 cells as an
indicator cell line. Sera collected from individual humans, rhesus monkeys, or

10   chimpanzees were inactivated at 56°C for 30 minutes. A serial dilution of each
sample (1:10, 1:20, 1:40, 1:80, 1:160, 1:320 in 100 µl of DMEM containing 10%
FCS) was added to equal amounts of H5.010CMVEGFP (1000 PFU/well) or
C68CMVEGFP virus and incubated at 4°C for two hrs. One hundred and fifty
microliters of the mixture were transferred onto 2 x 10 293 cells in 96 well flat bottom

15   plates. Control wells were infected with equal amounts of virus (without addition of
serum). Samples were incubated at 37°C in 5% CO_2 for 48 hrs and examined under a
fluorescent microscope. Sample dilutions that showed >50% reduction of green-
fluorescent foci as compared to infected controls were scored positive for neutralizing
antibodies.

20   As expected, approximately 35% of normal human subjects demonstrated
neutralizing antibody against Ad5, a frequency much higher than observed in sera of
rhesus monkeys and chimpanzee. Neutralizing antibody to C68 was observed in 80%
of chimpanzee and only 2% of normal human subjects or rhesus monkeys. Titers of
neutralizing antibodies in the non-target species were generally low.

25   To further evaluate cross-reactivity of C68 with human adenovirus vectors,
mice were immunized with 2 x 10^7 plaque forming units (pfu) of Ad 2, 4, 5, 7 and 12
as well as C68. Sera were harvested 2 weeks later and tested for antibodies that
neutralized either Ad5 or C68 vectors. Neutralizing antibody to Ad5 vector was only
detected in animals immunized with Ad5. Importantly, the only animals with

neutralizing antibody to C68 vector were those immunized with C68 vector; none of the human serotypes tested, including Ad4, generated antibodies in mice that neutralized C68 *in vitro*.

Important to the utility of C68 vector in human trials is the absence of neutralizing antibody in the human population. In our study, a screen of 50 normal human subjects failed to detect any significant neutralizing antibodies (>1:10) using the same assay that showed neutralizing antibodies in >50% of chimpanzees. Furthermore, sera of mice immunized with multiple human Ad serotypes including Ad4, did not neutralize infection with C68.

In another study, groups of ten to twenty ICR mice were vaccinated with varied doses of the Adhu5rab.gp or the AdC68rab.gp vaccine given subcutaneously (s.c.), intranasally (i.n.) or orally (per os). Mice were bled 21 days later and viral neutralizing antibody (VNA) titers expressed as international units were determined. Mice were challenged 4 weeks after vaccinated with 10 mean lethal doses of CVS-24 virus applied directly into the central nervous system.

	VNA Titers (% survival upon challenge)			
Vaccine Dose	5×10^7	5×10^6	5×10^5	5×10^4
Adhu5rab.gp,s.c.	972(100)	324 (100)	108(100)	12 (100)
AdC68rab.gp, s.c.	240 (100)	36 (100)	12(80)	8(80)
Adhu5rab.gp, i.n.	nt	162 (100)	162 (100)	18 (50)
AdC68rab.gp, i.n.	nt	54 (100)	162 (100)	18 (50)
	2×10^7	2×10^6	2×10^5	2×10^4
Adhu5rab.gp, per os	108 (100)	54 (88)	18 (80)	4(30)
AdC68rab.gp, per os	108 (100)	36 (78)	12 (55)	0.2(0)

These data demonstrate that the AdC68 construct unexpectedly induces a better protective antibody response at low doses intranasally than human type 5.

Example 15 - Structural analysis of hexon proteins

The absence of neutralizing antibodies between C68 and human serotypes compelled us to more carefully evaluate structural differences in the regions of hexon presumed to harbor type specific epitopes. Previous studies have suggested that these

5   epitopes are located within the 7 hypervariable regions of hexon determined by Crawford-Miksza and Schnurr (*J. Virol*, 70:1836-1844 (1996)). A comparison of the amino acid sequences of hexon proteins between C68 and several human adenoviruses is shown in Figure 2. Indeed, C68 is substantially dissimilar in significant regions of these hypervariable sequences. More detailed modeling of the

10   three dimensional structure of hexon of C68 was performed to map the unique sequences. Models of hexon structures from C68 and Ad4 were generated based on the x-ray crystal structures of hexons for Ad2 and Ad5.

The X-ray crystal structures of Ad5 hexon (Protein Data Bank identifier 1RUX) (J. J. Rux and R. M. Burnett, *Mol. Ther.* 1:18-30 (2000)), and that for Ad2 (F.

15   K. Athappilly, *et al*, *J. Mol. Biol.*, 242: 430-455 (1994)), have been further refined to yield the current hexon models (Rux and Burnett, to be published elsewhere). Models of the homologous C68 and Ad4 hexons were initially produced using the Swiss-PdbViewer protein-modeling environment (N. Guez and M.C. Peitsch, *Electrophoresis*, 18:2714-2723 (1997)). Its automated procedure was used to align

20   the C68 and Ad4 hexon amino acid sequences to those of the Ad2 and Ad5 hexon crystal structures. The sequence alignments were used to guide the threading of the model sequences onto the known molecular structures. The side chain positions of residues not seen in the known structures were selected from a library of side chain protomers. These initial molecular models were then manually adjusted to improve

25   the automated alignment by moving gaps to exposed variable regions and by optimizing the packing of side chains. The positions of external loop segments not observed in the Ad2 and Ad5 template structures were either selected from a library of known loop structures or fitted manually. The conformation of each model was

further refined by energy minimization using the molecular mechanics program
CHARMM (B. R. Brooks, et al, *J. Comp. Chem.*, 4:187-217 (1983)). The structures
of these C68 and Ad4 hexon models were then aligned and a new sequence alignment
calculated. The differences between the two structurally aligned hexon sequences

5   were used to color images of the homology models. Graphical images prepared
within the Swiss-PdbViewer program were exported and rendered with the
Persistence of Vision Ray Tracer program (POV-Ray 2000, Version 3.1g).

While the overall C68 sequence is very similar to that of Ad4 hexon, the
differences between the two sequences are primarily focused in the DE1 and FG1

10   loops, and these contain all seven hypervariable regions. It is the DE1, FG1, and FG2
loops, each from a different subunit, that intimately associate to form the tower
domains at the top of the trimeric molecule. The hexon towers form much of the
exterior surface of the virion and are the sites of antibody attachment. As the sides
and base of the hexons pack together within the capsid, these regions are shielded

15   from antibody binding and their sequences are conserved. In contrast, the sequences
of C68 and Ad4 are quite different in the hexon towers. This immediately explains
why antibodies raised to either of these viruses do not cross-react.

All publications cited in this specification, and the sequence listing, are
incorporated herein by reference. While the invention has been described with

20   reference to a particularly preferred embodiment, it will be appreciated that
modifications can be made without departing from the spirit of the invention. Such
modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1.   Use of a recombinant adenovirus in the preparation of a medicament for inducing a T cell response to an immunogen in a subject,

characterized in that said recombinant simian adenovirus preferentially induces a CD8+ T cell response to the immunogen when delivered subcutaneously, and

further characterized in that said recombinant adenovirus comprises a nucleic acid molecule encoding an immunogen under the control of regulatory sequences which direct expression of the immunogen in the subject.

2.   Use of a recombinant adenovirus in the preparation of a medicament for inducing an antibody response to an immunogen in a subject,

characterized in that said recombinant simian adenovirus preferentially induces an antibody response to the immunogen at low doses when delivered to the musosa, and

further characterized in that said recombinant adenovirus comprises a nucleic acid molecule encoding an immunogen under the control of regulatory sequences which direct expression of the immunogen in the subject.

3.   A method of preferentially inducing a CD8+ T cell response to an immunogen in a subject, said method comprising the step of delivering to the subject a recombinant simian adenovirus comprising a nucleic acid molecule encoding an immunogen under the control of expression control of regulatory sequences which direct expression of the immunogen in the subject.

4.   The method according to claim 3, wherein said recombinant simian adenovirus is delivered subcutaneously.

5.   The method according to claim 3 or claim 4, wherein said recombinant simian adenovirus is delivered at a low dose.

6.   The method according to any of claims 3 to 5, wherein said recombinant simian adenovirus is delivered at an effective dose which is between 10^4 pfu and 10^6 pfu.

7.   The method according to any of claims 3 to 6, wherein said recombinant simian adenovirus is a recombinant C68 chimpanzee adenovirus.

8.   The method according to any of claims 3 to 7, wherein the immunogen is selected from the group consisting of a peptide, polypeptide or protein is derived from a pathogenic virus selected from the group consisting of human immunodeficiency virus-1, human papilloma virus, and rabies.

9.   An immunogenic composition useful for inducing a cytolytic immune response for human immunodeficiency virus comprising (a) a recombinant *simian* adenovirus comprising an optimized nucleic acid sequence encoding a modified gag protein of human immunodeficiency virus-1 and (b) a physiologically compatible carrier.

10.   A method for inducing a CD8+ T cell response against human immunodeficiency virus in mammals comprising administering to the mammal the composition of claim 9.

11.   Use of an immunogenic composition according to claim 9 in preparing a medicament for inducing a CD8+ T cell response against human immunodeficiency virus in mammals.

12.   A method for inducing a CD8+ T cell response against human
papilloma virus in mammals comprising administering to the mammal
a recombinant simian adenovirus encoding an immunogenic protein derived from
human papilloma virus.

13.   Use of a recombinant simian adenovirus encoding an immunogenic
protein derived from human papilloma virus in preparing a medicament for inducing a
CD8+ T cell response against human papilloma virus in mammals.

14.   Use of a recombinant simian adenovirus encoding an immunogenic
protein derived from rabies virus in preparing a medicament for inducing a
neutralizing antibody response against rabies in mammals.

15.   A vaccine for human immunodeficiency virus comprising a
recombinant simian replication-defective adenovirus comprising an antigen of human
immunodeficiency virus-1 (HIV-1).

16.   The vaccine according to claim 15, wherein the antigen is selected
from among the group consisting of the envelope, pol, or gag regions of HIV-1.

17.   The vaccine according to claim 16, wherein the antigen comprises a
native antigen from which the genetic instability elements have been removed.

18.   The vaccine according to claim 16, wherein the antigen is HIV-1 gag
cDNA comprising the sequences of SEQ ID NO:6.

19.   The vaccine according to claim 15, wherein the adenoviral
recombinant is an E1-deleted adenovirus derived from a chimpanzee is derived from
chimpanzee strain 68.

20.   A vaccine for rabies comprising a recombinant simian replication-
defective adenovirus comprising a nucleic acid sequence encoding a rabies antigen
under the control of regulatory sequences which direct expression of the rabies
antigen.

21.   A method for inducing a CD8+ T cell response and protecting a
mammal against human immunodeficiency virus comprising administering to the
mammal the vaccine of claim 15.

Figure 1

FIG 2

Amino acid sequence alignment of adenovirus hexon proteins (C68–E, Ad4–E, Ad16–E, Ad3–B, Ad7–B, Ad2–C) showing hypervariable regions HVR1–HVR7.

HVR1

C68 – E	130	NTCQWTYKADGETA — TE — KTYTYGNAPVQGIN — ITKDGIQLGTD — T — DDQPI
Ad4 – E	130	NTCQW — K — DSDS — KMHTEGAAAMPGVTGKKIEADGEPRIDSTS — GTDTVI
Ad16 – E	130	NTCQW — K — DSDS — KMHTEGVAAMPGVTGKKIEADGEPGIDSTS — GTDTVI
Ad3 – B	130	NTSQWIVTTNGQNAVTT — TNTEGIASMKGN — ITKFEGQKDITTEGEEKPI
Ad7 – B	130	NTSQWIVTAGEERAVTT — TNTEGIASMKGDN — ITKLGEEGKDIT — ADNKPI
Ad2 – C	130	NSCEWEQTEDSGRAVAEDEEEEDEDEEEEEQNARDQATKKTHVYAQAPLSGET — ITKSGLQIGSDNAETQA — KPI

HVR2 **HVR3**

C68 – E	177	YADKTYQPEPQVGDAEWHDITGTDEKYGGRALKPDTKMKPCYGSFAKPTNKEGGQA — VK — TGTG
Ad4 – E	177	YADKTYQPEPQVGLDSWVDTNGAEKYGGRALKDTTKWNPCYGSFAKPTNKEGGQA — IK — DSEPTATTPNYDIDLAF
Ad16 – E	177	YADKTYQPEPQVGNASWVDANGTEKYGGRALKDTTKMKPCYGSFAKPTNKEGGQA — N — ILK — DSEAATTPNYDIDLAF
Ad3 – B	183	YADKTYQPEPQVGLESWTDTDGTNEKYGGRALKPATNMKPCYGSFARPTNIKGGQAKNRKVKPTTEGVETEEPDIDMEF
Ad7 – B	181	YADKTYQPEPQVGRESWTDTDGTNEKYGGRALKPATKMKPCYGSFAQPTNIKGGQAKNRKVKP — TEGDVETEEPDIDMEF
Ad2 – C	205	YADPSYQPEPQIGESQWNEDADA — N — AAGGRVLKKTTPMKPCYGSYARPTNPFGGQS — VLIVPD — EKGV — PLPKVDDQF

HVR4 **HVR5**

C68 – E	251	FDNRSAA — AAGLAPEIVLYTENVDLETPDTHIVYKAGTDDSSSSINLGQQAMPNRPNYIGFRDNFIGLMYYNSTGNM
Ad4 – E	253	FDSKII — VANYDPIVYTENVDLQTPDTHIVYKPGTEDISSESNLGQQAMPNRPNYIGFRDNFIGLMYYNSTGNM
Ad16 – E	253	FDNKNI — AANYDPDIVMYTENVDLTPDTHIVYKPGTEDJSSESNLGQQAMPNRPNYIGFRDNFIGLMYYNSTGNM
Ad3 – B	259	FDGRDAV — ACALAPEIVLYTENVLETPDSHVYKPETSN — NSHANLGQQAMPNRPNYIGFRDNFIGLMYYNSTGNM
Ad7 – B	130	FDGREA — ADAFSPEIVLYTENVNLETPDSHVYKPTISDDNSHANLGQQAKNRKVKP — FRDNFIGLMYYNSTGNM
Ad2 – C	277	FSNTISLNDRQGNATKPKVVLYSEDVNIETPDTHISYKPGKGDENSKAMLGQQSMPNRPNYIAFRDNFIGLMYYNSTGNM

HVR6 **HVR7**

C68 – E	327	GVLAGQASQLNAVVDLQDRNTELSYQLLLDSLGDRTRYFSMWNQAVDSYDPDVRIIENHGVEDELPNYCFPLDAVGRTDT — YQGIKANG — DQTTWTKDDS — VNDANEIGKGNPFAM
Ad4 – E	328	GVLAGQASQLNAVVDLQDRNTELSYQLLLDSLGDRTRYFSMWNQAVDSYDPDVRIIENHGVEDELPNYCFPLNVGLTDT — YQGVKVKTD — AG-S-E — KWDKDDTVSNANEIHVGNPFAM
Ad16 – E	328	GVLAGQASQLNAVVDLQDRNTELSYQLLLDSLGDRTRYFSMWNQAVDSYDPDVRIIENHGVEDELPNYCFPLNCVGFTDT — YQGVKVKTDAVAGTSG — IIQWDKDDTVSTANEIHGGNPFAM
Ad3 – B	338	GVLAGQASQLNAVVDLQDRNTELSYQLLLDSLGDRTRYFSMWNQAVDSYDPDVRIIENHGTEDELPNYCFPLNGIGPGHT — YQGIKVKTD — DTNGWEKDAN — VAPANEITIGNNTAM
Ad7 – B	334	GVLAGQASQLNAVVDLQDRNTELSYQLLLDSLGDRTRYFSMWNQAVDSYDPDVRIIENHGIGPAKT — YQGIKSK — D — NGWEKDDN — VSKSNEIAIGNNQAM
Ad2 – C	357	GVLAGQASQLNAVVDLQDRNTELSYQLLLDSIGDRTRYFSMWNQAVDSYDPDVRIIENHGTEDELPNYCFPLGGIGVTDT — YQGIKANGNGS-GDNGD — TIWTKDH — FATRNEIGVGNNFAM

SEQUENCE LISTING

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       The Trustees of The University of Pennsylvania
       Ertl, Hildegund C.J.
       Wilson, James M.

<120> Methods of Inducing a Cytotoxic Immune Response and Recombinant Simian Ad
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Arg Trp Glu Lys Ile Arg Leu Arg Pro Gly Gly Lys Lys Lys Tyr Lys
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Page 5

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6

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<213>   Artificial Sequence

<220>
<223>   Modified HIV-1 gag sequence

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Glu Lys Ile Arg Leu Arg Pro Gly Gly Lys Lys Lys Tyr Lys Leu Lys
            20   25   30

His Ile Val Trp Ala Ser Arg Glu Leu Glu Arg Phe Ala Val Asn Pro
        35   40   45

Gly Leu Leu Glu Thr Ser Glu Gly Cys Arg Gln Ile Leu Gly Gln Leu
    50   55   60

Gln Pro Ser Leu Gln Thr Gly Ser Glu Glu Leu Arg Ser Leu Tyr Asn
65   70   75   80

Thr Val Ala Thr Leu Tyr Cys Val His Gln Arg Ile Glu Ile Lys Asp
                85   90   95

Thr Lys Glu Ala Leu Asp Lys Ile Glu Glu Glu Gln Asn Lys Ser Lys
            100   105   110

Lys Lys Ala Gln Gln Ala Ala Ala Asp Thr Gly His Ser Asn Gln Val
        115   120   125

Ser Gln Asn Tyr Pro Ile Val Gln Asn Ile Gln Gly Gln Met Val His
    130   135   140

Gln Ala Ile Ser Pro Arg Thr Leu Asn Ala Trp Val Lys Val Val Glu
145   150   155   160

Glu Lys Ala Phe Ser Pro Glu Val Ile Pro Met Phe Ser Ala Leu Ser
                165   170   175

Glu Gly Ala Thr Pro Gln Asp Leu Asn Thr Met Leu Asn Thr Val Gly
                180   185   190

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Gly His Gln Ala Ala Met Gln Met Leu Lys Glu Thr Ile Asn Glu Glu
        195                 200             205

Ala Ala Glu Trp Asp Arg Val His Pro Val His Ala Gly Pro Ile Ala
    210                 215             220

Pro Gly Gln Met Arg Glu Pro Arg Gly Ser Asp Ile Ala Gly Thr Thr
225                 230             235                 240

Ser Thr Leu Gln Glu Gln Ile Gly Trp Met Thr Asn Asn Pro Pro Ile
            245                 250             255

Pro Val Gly Glu Ile Tyr Lys Arg Trp Ile Ile Leu Gly Leu Asn Lys
            260                 265             270

Ile Val Arg Met Tyr Ser Pro Thr Ser Ile Leu Asp Ile Arg Gln Gly
        275                 280             285

Pro Lys Glu Pro Phe Arg Asp Tyr Val Asp Arg Phe Tyr Lys Thr Leu
        290                 295             300

Arg Ala Glu Gln Ala Ser Gln Glu Val Lys Asn Trp Met Thr Glu Thr
305                 310             315                 320

Leu Leu Val Gln Asn Ala Asn Pro Asp Cys Lys Thr Ile Leu Lys Ala
            325                 330             335

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Val Gly Gly Pro Gly His Lys Ala Arg Val Leu
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<211>   311
<212>   PRT
<213>   Chimpanzee type adenovirus

<400>   8
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Lys Asp Gly Ile Gln Leu Gly Thr Asp Thr Asp Asp Gln Pro Ile Tyr
            35              40              45


Ala Asp Lys Thr Tyr Gln Pro Glu Pro Gln Val Gly Asp Ala Glu Trp
    50              55              60


His Asp Ile Thr Gly Thr Asp Glu Lys Tyr Gly Gly Arg Ala Leu Lys
65              70              75              80


Pro Asp Thr Lys Met Lys Pro Cys Tyr Gly Ser Phe Ala Lys Pro Thr
            85              90              95


Asn Lys Glu Gly Gly Gln Ala Asn Val Lys Thr Gly Thr Gly Thr Thr
            100             105             110


Lys Glu Tyr Asp Ile Asp Met Ala Phe Phe Asp Asn Arg Ser Ala Ala
        115             120             125


Ala Ala Gly Leu Ala Pro Glu Ile Val Leu Tyr Thr Glu Asn Val Asp
    130             135             140


Leu Glu Thr Pro Asp Thr His Ile Val Tyr Lys Ala Gly Thr Asp Asp
145             150             155             160


Ser Ser Ser Ser Ile Asn Leu Gly Gln Gln Ala Met Pro Asn Arg Pro
            165             170             175


Val Tyr Ile Gly Phe Arg Asp Asn Phe Ile Gly Leu Met Tyr Tyr Asn
            180             185             190


Ser Thr Gly Asn Met Gly Val Leu Ala Gly Gln Ala Ser Gln Leu Asn
        195             200             205


Ala Val Val Asp Leu Gln Asp Arg Asn Thr Glu Leu Ser Tyr Gln Leu
    210             215             220


Leu Leu Asp Ser Leu Gly Asp Arg Thr Arg Tyr Phe Ser Met Trp Asn
225             230             235             240


Gln Ala Val Asp Ser Tyr Asp Pro Asp Val Arg Ile Ile Glu Asn His
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10

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Gly Val Glu Asp Glu Leu Pro Asn Tyr Cys Phe Pro Leu Asp Ala Val
        260   265   270

Gly Arg Thr Asp Thr Tyr Gln Gly Ile Lys Ala Asn Gly Thr Asp Gln
        275   280   285

Thr Thr Trp Thr Lys Asp Asp Ser Val Asn Asp Ala Asn Glu Ile Gly
        290   295   300

Lys Gly Asn Pro Phe Ala Met
305   310

<210> 9
<211> 314
<212> PRT
<213> Human adenovirus type 4

<400> 9

Asn Thr Cys Gln Trp Lys Asp Ser Asp Ser Lys Met His Thr Phe Gly
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Ala Ala Ala Met Pro Gly Val Thr Gly Lys Lys Ile Glu Ala Asp Gly
            20   25   30

Leu Pro Ile Arg Ile Asp Ser Thr Ser Gly Thr Asp Thr Val Ile Tyr
        35   40   45

Ala Asp Lys Thr Phe Gln Pro Glu Pro Gln Val Gly Asn Asp Ser Trp
        50   55   60

Val Asp Thr Asn Gly Ala Glu Glu Lys Tyr Gly Gly Arg Ala Leu Lys
65   70   75   80

Asp Thr Thr Lys Met Asn Pro Cys Tyr Gly Ser Phe Ala Lys Pro Thr
                85   90   95

Asn Lys Glu Gly Gly Gln Ala Asn Leu Lys Asp Ser Glu Pro Ala Ala
            100   105   110

Thr Thr Pro Asn Tyr Asp Ile Asp Leu Ala Phe Phe Asp Ser Lys Thr
        115   120   125

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11

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Ile Val Ala Asn Tyr Asp Pro Asp Ile Val Met Tyr Thr Glu Asn Val
    130   135   140

Asp Leu Gln Thr Pro Asp Thr His Ile Val Tyr Lys Pro Gly Thr Glu
145   150   155   160

Asp Thr Ser Ser Glu Ser Asn Leu Gly Gln Gln Ala Met Pro Asn Arg
            165   170   175

Pro Asn Tyr Ile Gly Phe Arg Asp Asn Phe Ile Gly Leu Met Tyr Tyr
            180   185   190

Asn Ser Thr Gly Asn Met Gly Val Leu Ala Gly Gln Ala Ser Gln Leu
            195   200   205

Asn Ala Val Val Asp Leu Gln Asp Arg Asn Thr Glu Leu Ser Tyr Gln
    210   215   220

Leu Leu Leu Asp Ser Leu Gly Asp Arg Thr Arg Tyr Phe Ser Met Trp
225   230   235   240

Asn Gln Ala Val Asp Ser Tyr Asp Pro Asp Val Arg Ile Ile Glu Asn
            245   250   255

His Gly Val Glu Asp Glu Leu Pro Asn Tyr Cys Phe Pro Leu Asn Gly
            260   265   270

Val Gly Leu Thr Asp Thr Tyr Gln Gly Val Lys Val Lys Thr Asp Ala
            275   280   285

Gly Ser Glu Lys Trp Asp Lys Asp Asp Thr Thr Val Ser Asn Ala Asn
    290   295   300

Glu Ile His Val Gly Asn Pro Phe Ala Met
305   310

<210> 10
<211> 318
<212> PRT
<213> Human adenovirus type 16

<400> 10

Asn Thr Cys Gln Trp Lys Asp Ser Asp Ser Lys Met His Thr Phe Gly

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1                      5                          10                        15


Val Ala Ala Met Pro Gly Val Thr Gly Lys Lys Ile Glu Ala Asp Gly
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Leu Pro Ile Gly Ile Asp Ser Thr Ser Gly Thr Asp Thr Val Ile Tyr
            35                  40                  45


Ala Asp Lys Thr Phe Gln Pro Glu Pro Gln Val Gly Asn Ala Ser Trp
    50                  55                  60


Val Asp Ala Asn Gly Thr Glu Glu Lys Tyr Gly Gly Arg Ala Leu Lys
65                  70                  75                      80


Asp Thr Thr Lys Met Lys Pro Cys Tyr Gly Ser Phe Ala Lys Pro Thr
                85                  90                  95


Asn Lys Glu Gly Gly Gln Ala Asn Leu Lys Asp Ser Glu Thr Ala Ala
            100                 105                 110


Thr Thr Pro Asn Tyr Asp Ile Asp Leu Ala Phe Phe Asp Asn Lys Asn
            115                 120                 125


Ile Ala Ala Asn Tyr Asp Pro Asp Ile Val Met Tyr Thr Glu Asn Val
    130                 135                 140


Asp Leu Gln Thr Pro Asp Thr His Ile Val Tyr Lys Pro Gly Thr Glu
145                 150                 155                     160


Asp Thr Ser Ser Glu Ser Asn Leu Gly Gln Gln Ala Met Pro Asn Arg
                165                 170                 175


Pro Asn Tyr Ile Gly Phe Arg Asp Asn Phe Ile Gly Leu Met Tyr Tyr
            180                 185                 190


Asn Ser Thr Gly Asn Met Gly Val Leu Ala Gly Gln Ala Ser Gln Leu
            195                 200                 205


Asn Ala Val Val Asp Leu Gln Asp Arg Asn Thr Glu Leu Ser Tyr Gln
    210                 215                 220


Leu Leu Leu Asp Ser Leu Gly Asp Arg Thr Arg Tyr Phe Ser Met Trp
225                 230                 235                     240
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Asn Gln Ala Val Asp Ser Tyr Asp Pro Asp Val Arg Ile Ile Glu Asn
                245   250   255

His Gly Val Glu Asp Glu Leu Pro Asn Tyr Cys Phe Pro Leu Asn Gly
                260   265   270

Val Gly Phe Thr Asp Thr Tyr Gln Gly Val Lys Val Lys Thr Asp Ala
            275   280   285

Val Ala Gly Thr Ser Gly Thr Gln Trp Asp Lys Asp Asp Thr Thr Val
        290   295   300

Ser Thr Ala Asn Glu Ile His Gly Gly Asn Pro Phe Ala Met
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<213>   Human adenovirus type 3

<400>   11

Asn Thr Ser Gln Trp Ile Val Thr Thr Asn Gly Asp Asn Ala Val Thr
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Thr Thr Thr Asn Thr Phe Gly Ile Ala Ser Met Lys Gly Gly Asn Ile
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Thr Lys Glu Gly Leu Gln Ile Gly Lys Asp Ile Thr Thr Thr Glu Gly
        35   40   45

Glu Glu Lys Pro Ile Tyr Ala Asp Lys Thr Tyr Gln Pro Glu Pro Gln
    50   55   60

Val Gly Glu Glu Ser Trp Thr Asp Thr Asp Gly Thr Asn Glu Lys Phe
65   70   75   80

Gly Gly Arg Ala Leu Lys Pro Ala Thr Asn Met Lys Pro Cys Tyr Gly
            85   90   95

Ser Phe Ala Arg Pro Thr Asn Ile Lys Gly Gly Gln Ala Lys Asn Arg
            100   105   110

14

Lys Val Lys Pro Thr Thr Glu Gly Gly Val Glu Thr Glu Glu Pro Asp
        115   120   125

Ile Asp Met Glu Phe Phe Asp Gly Arg Asp Ala Val Ala Gly Ala Leu
    130   135   140

Ala Pro Glu Ile Val Leu Tyr Thr Glu Asn Val Asn Leu Glu Thr Pro
145   150   155   160

Asp Ser His Val Val Tyr Lys Pro Glu Thr Ser Asn Asn Ser His Ala
                165   170   175

Asn Leu Gly Gln Gln Ala Met Pro Asn Arg Pro Asn Tyr Ile Gly Phe
            180   185   190

Arg Asp Asn Phe Val Gly Leu Met Tyr Tyr Asn Ser Thr Gly Asn Met
        195   200   205

Gly Val Leu Ala Gly Gln Ala Ser Gln Leu Asn Ala Val Val Asp Leu
    210   215   220

Gln Asp Arg Asn Thr Glu Leu Ser Tyr Gln Leu Leu Leu Asp Ser Leu
225   230   235   240

Gly Asp Arg Thr Arg Tyr Phe Ser Met Trp Asn Gln Ala Val Asp Ser
                245   250   255

Tyr Asp Pro Asp Val Arg Ile Ile Glu Asn His Gly Ile Glu Asp Glu
            260   265   270

Leu Pro Asn Tyr Cys Phe Pro Leu Asn Gly Ile Gly Pro Gly His Thr
        275   280   285

Tyr Gln Gly Ile Lys Lys Val Lys Thr Asp Asp Thr Asn Gly Trp Glu
    290   295   300

Lys Asp Ala Asn Val Ala Pro Ala Asn Glu Ile Thr Ile Gly Asn Asn
305   310   315   320

Leu Ala Met

<210> 12

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<211>   315
<212>   PRT
<213>   Human adenovirus type 7

<400>   12

Asn Thr Ser Gln Trp Ile Val Thr Ala Gly Glu Glu Arg Ala Val Thr
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Thr Thr Thr Asn Thr Phe Gly Ile Ala Ser Met Lys Gly Asp Asn Ile
            20                  25                  30


Thr Lys Glu Gly Leu Glu Ile Gly Lys Asp Ile Thr Ala Asp Asn Lys
        35                  40                  45


Pro Ile Tyr Ala Asp Lys Thr Tyr Gln Pro Glu Pro Gln Val Gly Glu
    50                  55                  60


Glu Ser Trp Thr Asp Thr Asp Gly Thr Asn Glu Lys Phe Gly Gly Arg
65                  70                  75                  80


Ala Leu Lys Pro Ala Thr Lys Met Lys Pro Cys Tyr Gly Ser Phe Ala
            85                  90                  95


Arg Pro Thr Asn Ile Lys Gly Gly Gln Ala Lys Asn Arg Lys Val Lys
            100                 105                 110


Pro Thr Glu Gly Asp Val Glu Thr Glu Glu Pro Asp Ile Asp Met Glu
        115                 120                 125


Phe Phe Asp Gly Arg Glu Ala Ala Asp Ala Phe Ser Pro Glu Ile Val
    130                 135                 140


Leu Tyr Thr Glu Asn Val Asn Leu Glu Thr Pro Asp Ser His Val Val
145                 150                 155                 160


Tyr Lys Pro Gly Thr Ser Asp Asp Asn Ser His Ala Asn Leu Gly Gln
                165                 170                 175


Gln Ala Met Pro Asn Arg Pro Asn Tyr Ile Gly Phe Arg Asp Asn Phe
            180                 185                 190


Val Gly Leu Met Tyr Tyr Asn Ser Thr Gly Asn Met Gly Val Leu Ala
            195                 200                 205
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16

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Gly Gln Ala Ser Gln Leu Asn Ala Val Val Asp Leu Gln Asp Arg Asn
    210             215                 220

Thr Glu Leu Ser Tyr Gln Leu Leu Leu Asp Ser Leu Gly Asp Arg Thr
225             230                 235                 240

Arg Tyr Phe Ser Met Trp Asn Gln Ala Val Asp Ser Tyr Asp Pro Asp
                245                 250                 255

Val Arg Ile Ile Glu Asn His Gly Ile Glu Asp Glu Leu Pro Asn Tyr
            260                 265                 270

Cys Phe Pro Leu Asp Gly Ile Gly Pro Ala Lys Thr Tyr Gln Gly Ile
        275                 280                 285

Lys Ser Lys Asp Asn Gly Trp Glu Lys Asp Asp Asn Val Ser Lys Ser
    290                 295                 300

Asn Glu Ile Ala Ile Gly Asn Asn Gln Ala Met
305                 310                 315


<210>  13
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<212>  PRT
<213>  Human adenovirus type 2

<400>  13

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Glu Asp Glu Glu Glu Glu Asp Glu Glu Glu Glu Glu Glu Glu Glu Glu
            20                  25                  30

Gln Asn Ala Arg Asp Gln Ala Thr Lys Lys Thr His Val Tyr Ala Gln
        35                  40                  45

Ala Pro Leu Ser Gly Glu Thr Leu Thr Lys Ser Gly Leu Gln Ile Gly
    50                  55                  60

Ser Lys Asn Ala Glu Thr Gln Ala Lys Pro Val Tyr Ala Asp Pro Ser
65                  70                  75                  80

Tyr Gln Pro Glu Pro Gln Ile Gly Glu Ser Gln Trp Asn Glu Ala Asp
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                    85   90   95

Ala Asn Ala Ala Gly Gly Arg Val Leu Lys Lys Thr Thr Pro Met Lys
            100   105   110

Pro Tyr Gly Ser Tyr Ala Arg Pro Thr Asn Pro Phe Gly Gly Gln Ser
            115   120   125

Val Leu Val Pro Asp Glu Lys Gly Val Pro Leu Pro Lys Val Asp Leu
    130   135   140

Gln Phe Phe Ser Asn Thr Thr Ser Leu Asn Asp Arg Gln Gly Asn Ala
145   150   155   160

Thr Lys Pro Lys Val Val Leu Tyr Ser Glu Asp Val Asn Met Glu Thr
                165   170   175

Pro Asp Thr His Leu Ser Tyr Lys Pro Gly Lys Gly Asp Glu Asn Ser
            180   185   190

Lys Ala Met Leu Gly Gln Gln Ser Met Pro Asn Arg Pro Asn Tyr Ile
        195   200   205

Ala Phe Arg Asp Asn Phe Ile Gly Leu Met Tyr Tyr Asn Ser Thr Gly
    210   215   220

Asn Met Gly Val Leu Ala Gly Gln Ala Ser Gln Leu Asn Ala Val Val
225   230   235   240

Asp Leu Gln Asp Arg Asn Thr Glu Leu Ser Tyr Gln Leu Leu Leu Asp
            245   250   255

Ser Ile Gly Asp Arg Thr Arg Tyr Phe Ser Met Trp Asn Gln Ala Val
            260   265   270

Asp Ser Tyr Asp Pro Asp Val Arg Ile Ile Glu Asn His Gly Thr Glu
        275   280   285

Asp Glu Leu Pro Asn Tyr Cys Phe Pro Leu Gly Gly Ile Gly Val Thr
    290   295   300

Asp Thr Tyr Gln Ala Ile Lys Ala Asn Gly Asn Gly Ser Gly Asp Asn
305   310   315   320

                                  18

.

Gly Asp Thr Thr Trp Thr Lys Asp Glu Thr Phe Ala Thr Arg Asn Glu
              325   330   335

Ile Gly Val Gly Asn Asn Phe Ala Met
          340   345

.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 39/295
US CL : 424/199.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/199.1, 184.1, 204.1, 233.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 6,083,716 A (WILSON et al) 04 July 2000 (04.07.2000), see entire document, especially columns 15 and 16.	2, 14 ———— 1, 3- 13, 15- 21
Y	QIU et al. Evaluation of Novel Human Immunodeficiency Virus Type 1 Gag DNA Vaccines for Protein Expression in Mammalian Cells and Induction of Immune Responses. Journal of Virology. 1999, Vol. 73, No. 11 pages 9145- 9152, see entire document.	1, 3- 11, 15- 21
Y	TOES et al. Protective anti-tumor immunity induced by vaccination with recombinant adenoviruses encoding multiple tumor-associated cytotoxic T lymphocyte epitopes in a string-of-beads fashion. Proceedings of the National Academy of Sciences. U.S.A. 1997, Vol. 94, pages 14660- 14665, see abstract.	12 and 13
A,P	FARINA et al. Replication-Defective Vector Based on a Chimpanzee Adenovirus. Journal of Virology. 2001, Vol. 75, No. 23, pages 11603- 11613.	1- 21

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 July 2002 (29.07.2002)	04 OCT 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Myron G. Hill
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

Continuation of B. FIELDS SEARCHED Item 3:
WEST- US PAT, EPO, JPO, Derwent
Medline
terms- adenovirus, simian, chimpanzee, monkey, CD8, antibody, subcutaniously, HIV, Papilloma, strain 68